Biosynthesis of Lignans

Habilitationsschrift

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Attachments: Publications

Fuss E (2003): Lignans in plant cell and organ cultures: An overview. Phytochemistry Reviews 2, 307-320

Van Fürden B, Humburg A, **Fuss E** (2005): Influence of methyl jasmonate on podophyllotoxin and 6-methoxypodophyllotoxin accumulation in cell suspension cultures of *Linum album*. Plant Cell Reports 24, 312-317

von Heimendahl CBI, Schäfer K, Eklund P, Sjöholm R, Schmidt TJ, **Fuss E** (2005): Pinoresinol-lariciresinol reductases with different stereospecificity from *Linum album* and *Linum usitatissimum*. Phytochemistry 66, 1254-1263

Mohagheghzadeh A, Schmidt TJ, Bayindir Ü, **Fuss E**, Mehregan I, Alfermann AW (2006): Diarylbutyrolactone Lignans from *Linum corymbulosum in vitro* Cultures. Planta Medica 72, 1165-1167

Federolf K, Alfermann AW, **Fuss E** (2007) Aryltetralin-lignan formation in two different cell suspension cultures of *Linum album*: Deoxypodophyllotoxin 6-hydroxylase, a key-enzyme in the formation of 6-methoxypodophyllotoxin? Phytochemistry 68, 1397-1408

Hemmati S, Schmidt TJ, **Fuss E**: (2007) (+)-Pinoresinol/(-)-lariciresinol reductase from *Linum perenne* Himmelszelt involved in the biosynthesis of justicidin B. FEBS Letters 581, 603-610

1. Zusammenfassung

Lignane sind eine schon in Moosen und Farnen, vor allem aber in höheren Pflanzen vorkommende, weit verbreitete Klasse von Naturstoffen. Es handelt sich meist um Dimere aus Phenylpropaneinheiten, die über die jeweiligen *β*-Kohlenstoffatome der Seitenketten miteinander verknüpft sind. Weitere Derivatisierungen führen zu großer struktureller Diversität. Lignane kommen im Allgemeinen in der Pflanze als optisch aktive Verbindungen und nicht als Racemate vor. Sie besitzen vielfältige biologische Aktivitäten und dienen der Pflanze vermutlich als Abwehrstoffe gegen Fraßfeinde und Pathogene.

Das für den Menschen wichtigste pflanzliche Lignan ist sicherlich das Arylteralin-Lignan Podophyllotoxin (PTOX). Durch Bindung an Tubulin verhindert PTOX die Ausbildung funktionstüchtiger Mikrotubuli und dadurch auch die Zellteilung. Bis heute wird es äußerlich zur Behandlung von Feigwarzen (*Condylomata acuminata*) verwendet. In der Chemotherapie von Tumoren setzt man semisynthetische Podophyllotoxin-Derivate wie das Etopophos® ein. Diese binden nicht an Tubulin sondern hemmen die Topoisomerase II und verhindern dadurch ebenfalls die Zellteilung. Sie werden aus PTOX synthetisiert, das man aus den Wurzelstöcken von im Himalaya gesammelten Pflanzen von *Podophyllum hexandrum* isoliert. Die Bestände von *P. hexandrum* gelten inzwischen als gefährdet, weshalb man nach alternativen Rohstoffquellen sucht.

Als mögliche Alternativen bieten sich Pflanzen an, die ebenfalls PTOX enthalten. Als erstes wäre *Podophyllum peltatum* zu nennen, eine in Amerika beheimatete enge Verwandte von *P. hexandrum*, die jedoch in bestimmten Rassen den höchsten PTOX-Gehalt in den Blättern hat. Eine Kultivierung dieser Art ist bis heute ebenso schwierig wie die Kultivierung von *P. hexandrum*. Als weitere interessante Pflanzengruppe haben sich die Leingewächse (Linaceae) herausgestellt. In vielen Arten der Gattung *Linum* konnten PTOX und verwandte Strukturen wie insbesondere das 6-Methoxypodophyllotoxin (6MPTOX) nachgewiesen werden. Da aber auch die meisten Leinarten bis auf den Flachs (*Linum usitatissimum*), der allerdings nur einfachere Lignane wie das Secoisolariciresinoldiglucosid enthält, nicht angebaut werden, wurden Versuche mit pflanzlichen Zell- und Gewebekulturen durchgeführt. So konnten Van Uden und Mitarbeiter zeigen, dass Zellsuspensionskulturen von *Linum flavum* 6MPTOX akkumulieren. Die von uns etablierten Zellsuspensionslinien des Weißen Leins (*Linum album*), einer im Iran endemischen Pflanzenspezies, akkumulieren entweder fast keine Lignane (z. B. Linie X4SF) oder bis zu 0,8 % ihres Trockengewichts an PTOX (z. B. Linie PT) oder 6MPTOX (Linie 6M) als Hauptlignan in nur 10-12 Tagen.

Fernerhin haben wir Zellkulturen von *L. corymbulosum* etabliert, die ca. 360 µg/g Trockengewicht des Dibenzylbutyrolacton-Lignans Hinokinin (HINO) akkumulieren. Zellkulturen verschiedener Varietäten von *L. perenne* akkumulieren die Arylnaphthalin-Lignane Justicidin B (JusB) und verschiedene Glycoside des Diphyllins (Diph). Dabei akkumuliert die Kultur von *L. perenne* var. Himmelszelt mit 23 mg/g Trockengewicht die größte Menge JusB.

Wir wollen diese Kulturen nutzen, um die weitestgehend unbekannten Biosynthesewege der Lignanstrukturtypen zu untersuchen.

Über den Allgemeinen Phenylpropan- und Monolignolstoffwechsel werden die auch für die Biosynthese des Lignins benötigten Hydroxyzimtalkohole gebildet, aus denen durch Dimerisierung Lignane entstehen.
Ein hypothetischer Biosyntheseweg vom Coniferylalkohol zu PTOX und 6MPTOX ist in Abb. 4, S. 13 dargestellt. Die Reihenfolge der möglichen Intermediate wurde durch Fütterungsexperimente mit unmarkierten und markierten Substanzen an Pflanzenorgane oder Zellkulturen ermittelt. Die Biosynthese bis zur Stufe des Matairesinols wurde in der Arbeitsgruppe von Norman G. Lewis und Laurence B. Davin (Institute for Biological Chemistry, Washington State University, Pullman, USA) in *Forsythia*-Arten sowohl auf biochemischer als auch molekularbiologischer Ebene untersucht. Zwei Moleküle Coniferylalkohol werden zu Pinoresinol verknüpft. Dabei wirken als "Pinoresinol-Synthase" ein radikalbildendes Enzym (vermutlich eine Laccase) und ein sogenanntes „dirigent protein“ zusammen. Letzteres hat keine eigene enzymatische Funktion bei der Dimerisierung. Es orientiert die Coniferylalkoholradikale aber so zueinander, dass nur ein Enantiomer des Pinoresinols entsteht. In den nächsten beiden Schritten katalysiert die Pinoresinol-Lariciresinol-Reduktase (PLR) die Umwandlung des Pinoresinols zu Lariciresinol und Secoisolariciresinol. Letzteres wird von der Secoisolariciresinol-Dehydrogenase zu Matairesinol umgewandelt. Auch diese Reduktions- und Oxidationsschritte laufen zumindest teilweise stereospezifisch ab.
Die weiteren Schritte in der Biosynthese des PTOX und 6MPTOX sind bisher kaum biochemisch und gar nicht molekularbiologisch untersucht. Die Schritte zwischen Matairesinol und Yatein wurden erst kürzlich durch Einbaustudien markierter möglicher Intermediate mit Sprossen von *Anthriscus sylvestris*, einer guten Quelle für Yatein, in der Arbeitsgruppe von T. Umezawa untersucht. Dewick (1986) zeigte den Einbau von radioaktiv markiertem Yatein in PTOX und ebenso von Desoxypodophyllotoxin (DOP) in PTOX.
Biotransformationsversuche mit Suspensionskulturen von *Linum flavum* und *Podophyllum hexandrum* lieferten wichtige Informationen über die biosynthetischen Zusammenhänge der Podophyllotoxinderivate. DOP wurde von *Podophyllum*-Zellen, die hauptsächlich PTOX akkumulieren, ausschließlich zu PTOX, von *L. flavum*-Zellkulturen mit 6MPTOX als Hauptlignan jedoch zu 6MPTOX-β-D-Glucosid umgewandelt (Van Uden et al.). *L. flavum* verwandelte darüber hinaus zugefüttertes PTOX in PTOX-β-D-Glucosid, und nicht zu 6MPTOX (bzw. dessen β-D-Glucosid), zugefüttertes β-Peltatin dagegen zu β-Peltatin- und 6MPTOX- β-D-Glucosid. Dies zeigt, dass wahrscheinlich die Einführung einer ersten OH-Gruppe in DOP die Weichen stellt: 7-Hydroxylierung führt ausschließlich zum Aglycon PTOX, 6-Hydroxylierung dagegen zu den Aglyca β-Peltatin und 6MPTOX. Analoge Experimente mit den *L. album* Zellinien 6M und PT konnten diese Aussage bestätigen. Auf enzymatischer Ebene konnten bisher neben den Schritten von Coniferylalkohol zu Matairesinol nur einige der letzen Schritte der Biosynthese des 6MPTOX gezeigt werden. Eine Cytochrom P450-abhängige Monooxygenase sowie eine Methyltransferase katalysieren die Überführung von DOP in β-Peltatin und anschließend in Peltatin-A Methylether. Nachdem unsere Versuche, die Aktivität der Desoxypodophyllotoxin-7-Hydroxylase, die DOP zu PTOX umsetzt, in zellfreien Extrakten nachzuweisen, gescheitert waren, unternahmen wir Fütterungsexperimente, um den Typ des beteiligten Enzyms nachzuweisen. Dabei kommen vor allem

Cytochrom P450 Monooxygenasen und Dioxygenasen in Frage. Die Gabe von Cytochrom P450 Monooxygenase Inhibitoren vor Zugabe von DOP führte in Linie 6M zur Reduktion der Akkumulation von Peltatin-A-Methylether aber zur Steigerung der PTOX Akkumulation. Daraus ist zu schließen, dass es sich bei der Desoxypodophyllotoxin-7-Hydroxylase wahrscheinlich nicht um eine Cytochrom P450 Monooxygenase handelt. PTOX wird immer nur dann in größerer Menge gemacht, wenn die Desoxypodophyllotoxin-6-Hydroxylase-aktivität, die den Eingang zur Biosnthese des 6MPTOX darstellt, fehlt. Daher liegt die Vermutung nahe, dass die Peltatin-A-Methylether-7-Hydroxylase, die normalerweise Peltatin-A-Methylether zu 6MPTOX umwandelt, bei Mangel ihres eigentlichen Substrats als Desoxypodophyllotoxin-7-Hydroxylase wirkt und Desoxypodophyllotoxin an Position 7 zu PTOX hydroxyliert.

Unter anderem als Voraussetzung für molekularbiologische Untersuchungen zur Biosynthese von PTOX/6MPTOX haben wir cDNA-Banken der *L. album* Zellkulturen 6M und PT hergestellt. Mithilfe dieser Banken wurden cDNAs für fast alle Schritte des Allgemeinen Phenylpropanstoffwechsels und eine cDNA für die PLR isoliert und in *E. coli* heterolog exprimiert. Die meisten Proteine wurden nach Reinigung bis zur Homogenität biochemisch charakterisiert. Wir möchten die fehlenden Gene für Enzyme und Regulatoren der PTOX/6MPTOX-Biosynthese durch einen cDNA-AFLP-Ansatz, der eine differentielle Genexpression zur Grundlage hat, klonieren. Dazu stehen zwei differentielle Systeme zur Verfügung. Verschiedene Zellkulturen von *L. album* akkumulieren entweder kaum Lignane, PTOX oder 6MPTOX als Hauptlignan (siehe oben). Wir konnten die Akkumulation von PTOX und 6MPTOX durch Zugabe von Analogen des Elicitors Coronatin und/oder Methyljasmonat erheblich steigern. Die höchste PTOX Menge von bis zu 250 mg/l wurde nach Zugabe von Coronalon beobachtet. Das ist die größte Menge PTOX, die bisher mit Zellkulturen erreicht wurde. Enzymaktvitäten und mRNA Spiegel von Enzymen der Lignanbiosynthese sind in den differentiellen Systemen unterschiedlich hoch, was sie zu einem geeigneten System für die cDNA-AFLP macht.

Die Biosynthese von HINO und JusB bzw. den Diph-Glycosiden war zu Beginn unserer Arbeiten weder biochemisch noch molekularbiologisch untersucht. Es konnten lediglich hypothetische Biosynthesewege aufgestellt werden (Abb. 9 und 11, S. 21, 22).

In der Biosynthese des HINO sind grundsätzlich zwei Wege vorstellbar. Zum einen kann die Biosynthese von Pinoresinol ausgehend über eine PLR als Eingangsenzym über Matairesinol zu HINO führen. Zum anderen käme ein Weg über Sesamin als Zwischenstufe in Frage. Wir konnten aus einer cDNA Bank der HINO akkumulierenden Zellkultur von *L. corymbulosum* eine cDNA für eine PLR isolieren. Das heterolog exprimierte und gereinigte Protein ist enantiospezifisch für die Umwandlung von (+)-Pinoresinol zu (-)-Secoisolariciresinol, was zur Akkumulation des HINO als (-)-Enantiomer passt. Experimente zur Suppression der *plr* Genexpression sollen den Beweis liefern, dass diese PLR an der HINO Biosynthese in *L. corymbulosum* beteiligt ist. Damit wäre wahrscheinlich der Weg über Matairesinol der richtige Weg zur Biosynthese des HINO. Zum weiteren Nachweis wollen wir aber noch versuchen, cDNAs für weitere Schritte beider Biosynthesewege zu klonieren. Ausserdem soll durch Ausweitung der Versuche auf weitere HINO führende Pflanzen wie

Chamaecyparis obtusa gezeigt werden, ob die für *L. corymbulosum* gewonnenen Erkenntnisse auf andere Species übertragbar sind.

Als Einstieg zum Verständnis der JusB/Diph Biosynthese haben wir eine cDNA für eine PLR aus der *L. perenne* var. Himmelszelt Suspensionskultur isoliert. Das heterlog exprimierte Protein bevorzugt im ersten Reaktionschritt (+)-Pinoresinol, im zweiten aber (-)-Lariciresinol. Damit ist zum ersten Mal eine PLR kloniert worden, die in den beiden Reaktionschritten entgegengesetzte Enantioselektivität zeigt. Durch RNAi-Experimente konnten wir nachweisen werden, dass die PLR an der Biosynthese von JusB und den Diph-Glycosiden beteiligt ist. Zudem konnten wir die Umsetzung von JusB zu Diph in zellfreien Extrakten der *L. perenne* var. Himmelszelt Zellkultur nachweisen. Versuche mit Inhibitoren für Cytochrom P450 Monooxygenasen (Cytochrom c und Clotrimazol) zeigen, dass die Hydroxylierung des JusB zu Diph an Position 7 von einer Cytochrom P450 abhängigen Monooxygenase katalysiert wird.

Wir haben ein Transformationsprotokoll zur genetischen Manipulation der Lignanbiosynthese in Leinarten mithilfe von *Agrobacterium rhizogenes*, welcher den Hairy Root Phänotyp hervorruft, etabliert. Dabei dienen Sprosskulturen, die jeweils auf nur einen Keimling, also nur einen Genotypen zurückgehen, als Ausgangsmaterial, um die Variabilität der Grundgehalte an Lignanen möglichst gering zu halten. Dies ist die Vorausetzung, um Effekte von Überexpression oder Suppression von Genen, die in der Lignanbiosynthese beteiligt sind, verfolgen zu können. Hairy Roots von *L. album* akkumulieren im Durchschnitt 40 +/- 3 mg/g Trockengewicht 6MPTOX., Hairy Roots von *L. corymbulosum* 0.28 +/- 0.00 mg/gTrockengewicht HINO, die von *L. perenne* var. Himmelszelt die schon in Zellkulturen nachgewiesenen Diph-Glycoside und 36 +/- 2 mg/g Trockengewicht JusB. Experimente mit RNAi-Konstrukten zur Suppression der PLR-Genexpression in *L. album* und *L. perenne* var. Himmelszelt konnten erstmals die Beteiligung der jeweiligen PLRs an der Biosynthese des 6MPTOX in *L. album* und des JusBs bzw. der Diphyllinglycoside in *L. perenne* var. Himmelszelt beweisen. Das von uns etablierte Protokoll eröffnet die Möglichkeit, genetisch in die Lignanbiosynthese einzugreifen. Neu klonierte Gene (z. B. über cDNA-AFLP, siehe oben) können so auf ihre Beteiligung in der Lignanbiosynthese überprüft werden.

Neben den beschriebenen Untersuchungen zur Aufklärung verschiedener Lignanbiosynthesewege, liegt ein weiterer Schwerpunkt unserer Arbeit auf dem Verständnis der Stereochemie der Lignane. Die meisten Lignane sind chirale Moleküle. In einer Pflanze oder einem Pflanzenorgan kommt meist nur ein Enantiomer eines Lignans vor. Die Enantiomerenreinheit scheint während der Biosynthese auf verschiedenern Ebenen festgelegt zu werden. In *Forsythia* species führt schon die Kopplung der beiden achiralen Coniferylalkoholmoleküle durch die Anwesenheit eines „dirigierenden Proteins“, welches selbst nicht katalytisch aktiv ist, zu reinem (+)-Pinoresinol. In *Wikstroemia sikokiana* wird Enantiomerenreinheit erst auf Ebene des Matairesinols erreicht.

Wir konnten feststellen, dass Ennatiomerenreinheit in Leinarten durch enantiospezifische PLRs festgelegt wird. So liegt Pinoresinol in Zellkulturen und „Hairy Roots“ von *L. album* als Gemisch beider Enantiomere vor, Secoisolariciresinol aber als reines (-)-Enantiomer. Proteinextrakte aus „Hairy Roots“ und die heterolog exprimierte PLR aus *L. album* zeigen hohe Enantioselektivität für (+)-Pinoresinol und Spezifität für die Bildung von (-)-Secoiso-

lariciresinol. Zellkulturen von *L. usitatissimum* enthalten ebenfalls ein Gemisch beider Pinoresinol-Enantiomere im Gegensatz zu *L. album* aber das andere Enantiomer des Secoisolariciresinol, nämlich das (+)-Secoisolariciresinol. In Samen von *L. usitatissimum* liegt zu 99 % (+)-Secoisolariciresinoldiglucosid, aber zu 1 % auch das andere Enantiomer vor (Davin und Lewis 2003). In blühenden Pflanzen von *L. usitatissimum* konnten Lignane mit Konfiguration wie die in *L. album* gefunden werden. Es konnten zwei PLRs von *L. usitatissimum* mit entgegengesetzter Enantiospezifität kloniert werden. In Blättern blühender Pflanzen war nur die Expression der für die Bildung von (-)-Secoisolariciresinol spezifischen PLR nachweisbar. Dagegen werden in sich entwickelnden Samen die Transkripte beider PLRs gefunden.

Um die molekularen Ursachen der Unterschiede in der Enantiospezifität von PLRs zu bestimmen, wurden Sequenzvergleiche von mehreren PLRs entgegengesetzter Enantiospezifität vorgenommen. So konnten drei Aminosäurepositionen identifiziert werden, bei denen in (+)-Pinoresinol spezifischen PLRs eine andere Aminosäure konserviert ist als in (-)-Pinorersinol spezifischen. Der Vergleich mit der Kristallstruktur einer (-)-Pinoresinol spezifischen PLR von *Thuja plicata* (Min et al. 2003) erhärtete die Vermutung, dass diese Positionen für die Enantiospezifität verantwortlich sind. Durch Austausch dieser drei Aminosäuren in der PLR von *L. album* erreichten wir die nahezu vollständigen Umkehr der Enantiospezifität. Damit konnte die Beteiligung dieser Aminosäuren an der Enantiospezifität von PLRs gezeigt werden.

Die PLR gehört zur Familie der PIP-Reduktasen, abgeleitet von ***P***inoresinol-Lariciresinol-Reduktase, ***I***soflavon-Reduktase (IFR) und ***P***henylcumaranbenzylether-Reduktase (PCBER), Enzymen, die sich nicht nur durch ihre hohe Sequenzähnlichkeit sondern auch ähnliche Reaktionsmechanismen auszeichnen (Davin und Lewis). Gang et al. schlugen vor, dass die nur regiospezifischen PCBERs die Vorläufer der enantiospezifischen PLRs und IFRs sind. Um tieferen Enblick in die Evolution der PLRs und ihre Enantiospezifität zu erlangen, haben wir einen phylogenetischer Stammbaum aufgestellt. Dazu wurden die in der Literatur und in Datenbanken identifizierten Reduktasen mit Ähnlichkeit zu PLRs herangezogen, aber auch weitere von uns klonierte PIP-Reduktasen aus *Linum* species und *Arabidopsis thaliana*.

Wir haben 8 Mitglieder der PIP-Familie im Genom von *A. thaliana* identifiziert. Die Einordnung der Proteine in einen Stammbaum (Abb. 36, S. 53) und ihre heterologe Expression der Proteine mit Funktionalitätsprüfung ergab, dass es sich bei AT 1 und AT2 um PLRs handelt. PLR-AT1 bevorzugt weder (+)-noch (-)-Pinoresinol, zeigt aber eine leichte Bevorzugung von (-)-Lariciresinol gegenüber von (+)-Lariciresinol. PLR-AT2 bevorzugt im ersten Schritt (-)-Pinoresinol und im zweiten Schritt (-)-Lariciresinol, zeigt also wie die PLR aus *L. usitatissimum* die bevorzugte Bildung von (+)-Secoisolariciresinol. AT3 ist eine PCBER. Das Protein hat aber auch PLR-Aktivität, wobei bevorzugt (+)-Pinoresinol verbraucht wird. AT6 und AT7 zeigen weder gute PLR noch gute PCBER-Aktivität, gehören aber im phylogenetischen Stammbaum ebenso wie AT4 und AT5, die kaum oder gar nicht in *E. coli* in löslicher Form exprimiert werden können, zur Gruppe der PCBER-artigen. Zwei weitere Proteine, VRL (Vestitonereduktase-ähnliches Protein, AT9) und DVR (3,8-Divinylprotochlorophyllide *a*-8-Vinyl-Reduktase, AT10), zeigen nur noch schwache Ähnlichkeit zu PLRs, sie stehen in der Stammbaumanalyse abseits.

Wie man im phylogenetischen Baum sieht, kann die PIP-Familie um folgende Enzyme erweitert werden: Pterocarpanreductasen (PTR), wie sie in der Biosynthese von Isoflavonen beteiligt sind; Eugenolsynthasen (EGS) und Isoeugenolsynthasen (IGS), jüngst klonierte Enzyme, die Coniferylalkoholacetat als Substrat verwenden; Leucoanthocyanidin-Reduktasen (LARs) der Flavonoidbiosynthese. Daneben besteht noch entfernte Ähnlichkeit zu 3,8-Divinylprotochlorophyllide *a*-8-Vinyl-Reduktasen (DVRs) und UDP-Glucose/Galaktose-4-Epimerasen (GALEs), die im Kohlenhydratstoffwechsel der Pflanzen aber auch anderer Organismen beteiligt sind. Letztere könnten die Ursprünge der PIP-Familie darstellen.
GALE, VRL-AT9 und DVR können als „Outgroup“ angesehen werden. LARs bilden ein eigenes Cluster. Die übrigen Enzyme bilden zwei Obercluster, die jeweils in 2 weitere Untercluster unterteilt sind. Ein Obercluster enthält das Untercluster der PLRs und das allerdings nicht so gut unterstützte Untercluster aus IGS und EGS. Das weitere Obercluster enthält ein Untercluster der PCBERs, in dem aber auch die PTRs vertreten sind, und das gut abgetrennte Untercluster der IFRs. Dies würde bedeuten, dass LARs und alle anderen einen gemeinsamen Vorläufer hatten. Dann haben sich die Gruppen der PLR/IGS/EGS und der PCBER/IFR entwickelt, die sich jeweils in die PLR und IGS/EGS und PCBER und IFR aufgespalten haben. Interessanterweise ist die Gruppe der PLRs in zwei Cluster aufgeteilt. Ein Cluster enthält nur PLRs aus Coniferopsida, das andere nur PLRs aus Magnoliopsida. Weiterhin auffällig ist die Aufteilung der Magnoliopsida-PLRs in zwei Cluster. Das eine enthält PLRs mit Enantiospezifität für (+)-Pinoresinol, bis auf die noch partielle Sequenz der neu klonierten PLR aus *L. usitatissimum*, deren Enantiospezifität noch nicht eindeutig gezeigt ist. Im anderen Cluster sind PLRs mit großer Variation der Enantiospezifität vertreten, unter anderem auch die für (-)-Pinoresinol-spezifische zuerst klonierte PLR aus *L. usitatissimum*. Da die beiden PLRs aus *L. usitatissimum* jeweils näher mit anderen Leinarten und weiteren Pflanzenarten als untereinander verwandt sind, müssen schon zwei PLR-Gene vor der Aufspaltung der Gattung in die zahlreichen Arten vorhanden gewesen sein. Analoges gilt für die PLRs der Coniferopsida. Aus der deutlichen Trennung der PLR-Cluster von Magnoliopsida und Coniferopsida, lässt sich schließen, dass bei deren Entwicklung jeweils eine Verdopplung eines PLR-Gens stattgefunden hat. Da eine klare Trennung von PCBERs und PLRs sowohl aus Magnoliopsida als auch Coniferopsida zu erkennen ist, und da PLRs und PCBERs in einer Pflanzenspecies gemeinsam gefunden werden, muss man annehmen, dass die Entstehung von PLRs und PCBERs schon in den Vorfahren dieser Klassen stattgefunden hat. Demzurfolge ist der Vorläufer dieser Enzyme in ursprünglicheren Klassen zu suchen. Dieser Vorläufer könnte schon Enantiospezifität gezeigt haben, da wir zumindest in der PCBER AT3 aus *Arabidopsis thaliana* in der Umsetzung von Pinoresinol eine Bevorzugung für das (+)-Enantiomer feststellen konnten. Dies könnte bedeuten, dass die Enantiospezifität der PLR-ähnlichen Proteine eine phylogenetisch alte Erfindung ist. Andererseits zeigt die Variabilität der Enantiospezifität der PLRs in der Gruppe PLR-AT1, PLR-AT2, PLR-Lp1 und PLR-Lu1, von nicht enantiospezifisch (PLR-AT1) bis hin zu hoch enantiospezifisch (PLR-Lu1), dass die Enantiospezifität im Zuge der Evolution nicht nur verändert, sondern auch ganz verloren gehen kann. Dies widerspricht Aussagen von Gang et al. (1999), die PCBERs aufgrund des Fehlens von Enantiospezifität als phylogenetische Vorläufer von enantiospezifisch arbeitenden PLRs und IFRs annehmen.

Betrachtet man jetzt nur die PLRs aus Leinarten, ist festzustellen, dass bei der Spezifizierung zu den Leinarten wahrscheinlich eine Verdopplung des im Genom des „Leinurahns“ vorhandenen PLR-Gens stattgefunden hat. Wohingegen eine für 8R,8’R-konfiguriertes Pinoresinol spezifische Form ihre Enantiospezifität im Laufe der Evolution erhalten hat, zeigen sich aus der zweiten Genkopie entwickelte PLRs eine hohe Variabilität der Enantiospezifität. Diese Aussagen müssen aber noch durch das Ergänzen des Stammbaums mit weiteren Lein-PLRs erhärtet werden.

In Pflanzen oder Organen von Leinarten kommen nur komplexere Lignane vor, wenn (-)-Secoisolariciresinol [(-)-Seco] als Intermediat vorliegt. So enthalten blühende Pflanzen von *L. usitatissimum* Lignane bis zur Stufe des (-)-Yatein, dem (-)-Seco als Vorstufe vorausgeht. Samen enthalten dagegen nur (+)-Seco-glucosid und wenig (+)-Matairesinol. Um zu klären, ob die Enzyme zur Synthese komplexer Lignane in Leinarten nur (-)-Seco oder seine Derivate als Substrat akzeptieren, wird die Lignanbiosynthese in Leinen so genetisch manipuliert, dass (-)-Seco als Vorstufe vorliegt. Dazu sollen in zukünftigen Experimenten z. B. die Samen-eigenen Eingangsenzyme (evtl. dirigierendes Protein und PLR) der Lignanbiosynthese in Samen von *L. usitatissimum* durch die in blühenden Pflanzen ersetzt werden und umgekehrt. Die Frage wäre dann, ob in den mutierten Pflanzen in Samen aus (-)-Seco als Vorstufe komplexerer Lignane gebildet werden können und ob in blühenden Pflanzen mit (+)-Seco als Vorstufe keine Bildung komplexerer Lignane mehr möglich ist.

2. General Introduction

2.1. Lignans and their function in plants and for human health

Lignans are widespread in the plant kingdom. They can be already found in mosses and ferns (Stafford, 2000). Most lignans are dimers of phenylpropanoid units which are linked via their β-carbon atoms (Fig. 1). Dimers of phenylpropanoid units which are coupled via other linkages are named neolignans (IUPAC, Moss 2000).

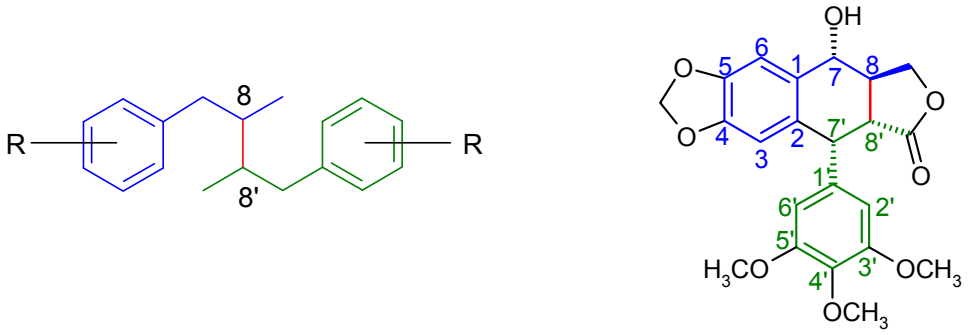


Fig. 1: left: general structure of lignans, right: podophyllotoxin with the numbering of the C-atoms according to IUPAC (Moss 2000)

Further derivatisation of this general structure leads to a broad variety of derivatives (Fig. 2). In addition, most lignans contain chiral C-atoms. Other natural compounds with chiral centers usually occur only in one enatiomeric form. In contrast, lignans can be found in both forms in different plant species or organs of the same species (Fig. 2) (Umezawa 2003).
Since lignans can show e.g. antiviral, fungicidal, antibacterial and cytotoxic activities they are thought to be involved in the plant defence against pathogens and are important for human health. The lignan secoisolariciresinoldiglucoside which is found in high amounts in seeds of flax (*Linum usitatissimum*) is converted by intestinal bacteria into the phytoestrogens enterodiol and enterolactone which protect against e.g. prostate and breast cancer (Rickard-Bon and Thompson 2003). The most important lignan for human health is probably the cytotoxic aryltetralin lignan podophyllotoxin (PTOX) because its semisynthetic derivatives like etopophos® are used in cancer therapy (Imbert 1998).

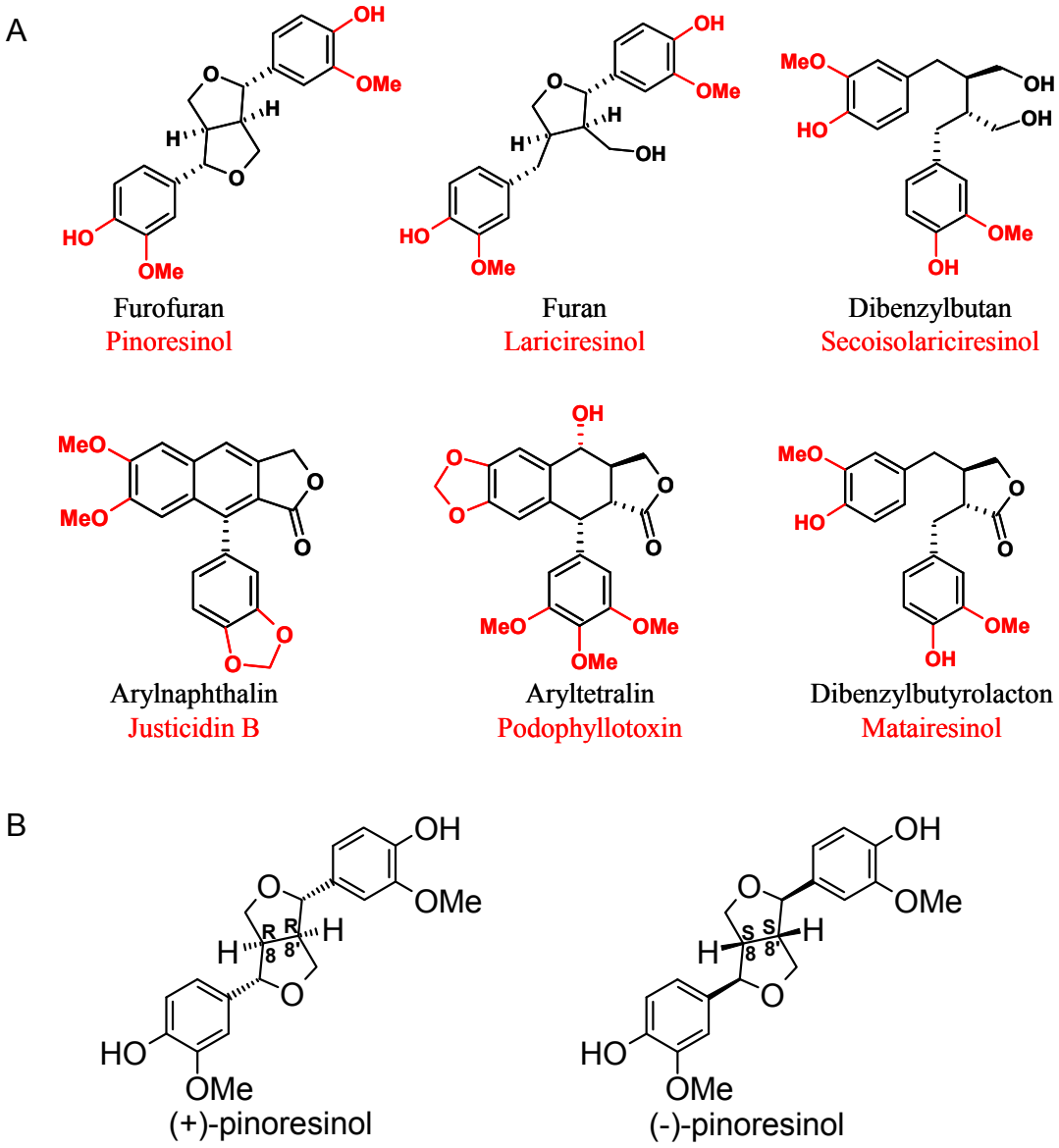


Fig. 2: Variations of the general lignan structure
A: Due to further derivatisation of the general structures
B: Due to stereo chemical conformations

2.2. Biosynthesis of lignans

The precursors for lignan biosynthesis, the monolignols, especially coniferyl alcohol, are formed in the general phenylpropanoid and the monolignol pathway (Fig. 3) (Humphreys and Chapple, 2002).

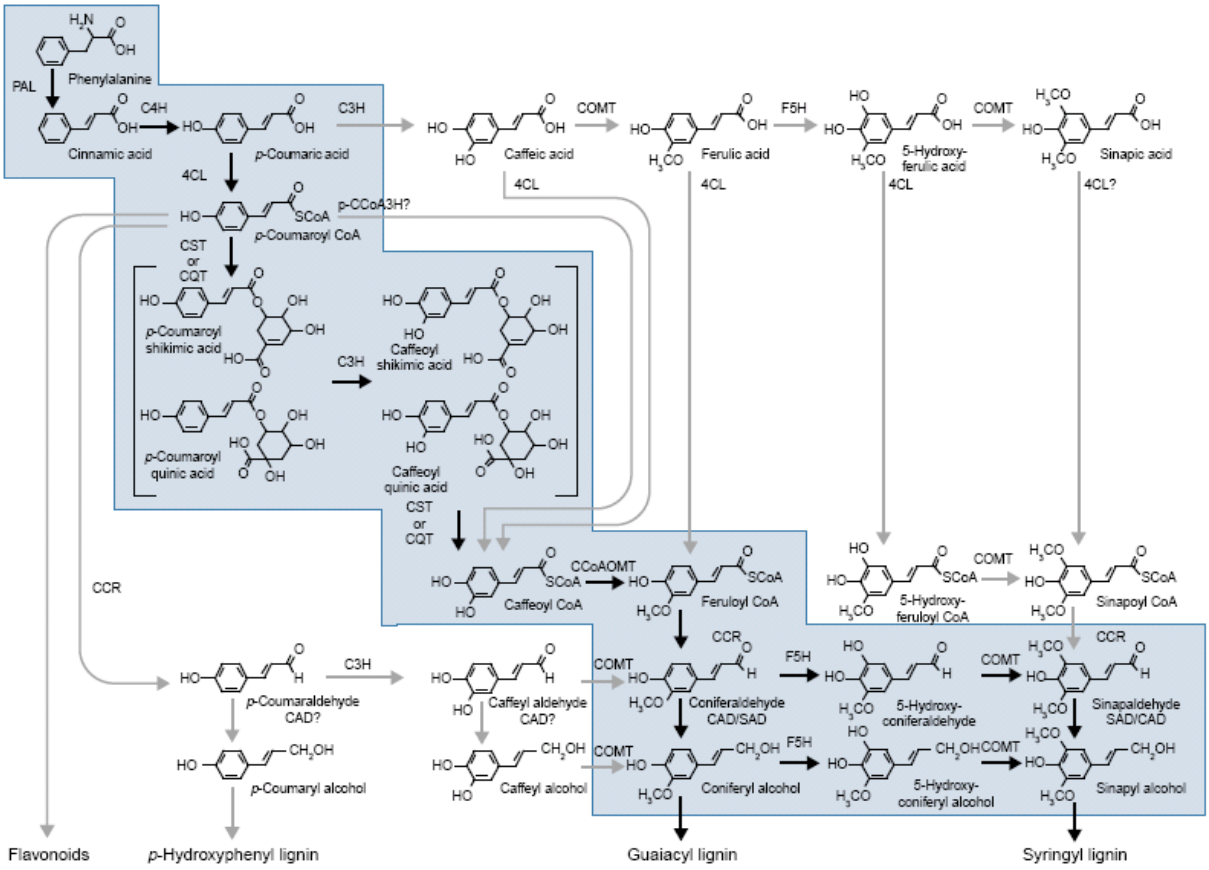


Fig. 3: General phenylpropanoid and monolignol pathway (Humphreys and Chapple, 2002)

Less is known about the specific steps leading to the biosynthesis of complex lignan structures like PTOX and JusB.

During the last 20 years most work was performed to understand the biosynthesis of podophyllotoxin and the related 6-methoxypodophyllotoxin (6MPTOX). A hypothetical biosynthetic pathway leading to PTOX and 6MPTOX could be developed by feeding of possible precursors (Fig. 4) (Rahman et al. 1990; Broomhead and Dewick 1991; Van Uden et al. 1995, Sakakibara et al. 2003, Fuss, unpublished results).

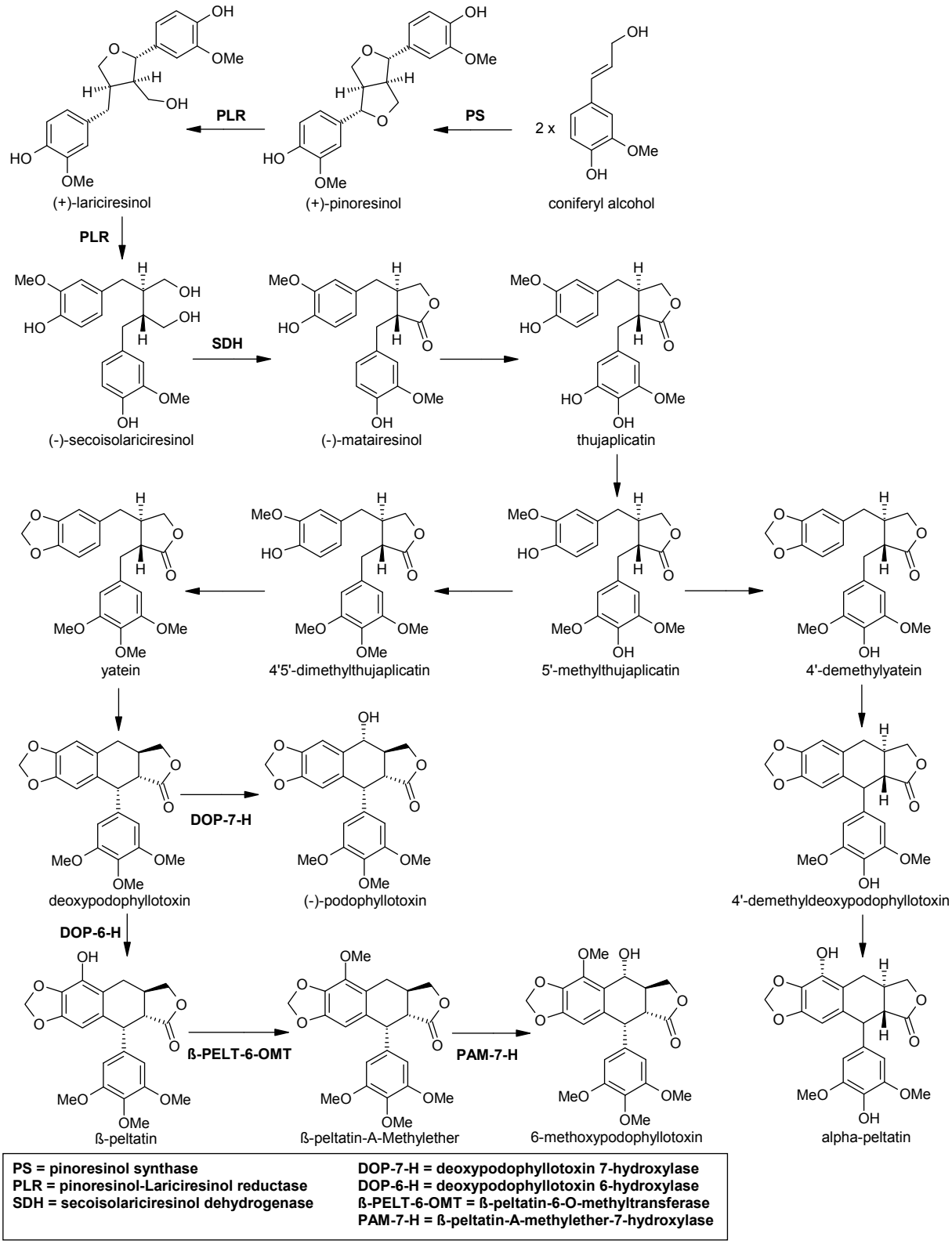


Fig. 4: Hypothetical biosynthetic pathway of podophyllotoxin and related lignans (Fuss, unpublished)

Up to now only cDNAs of the first biosynthetic steps were cloned (Davin and Lewis 2003). Two molecules of coniferyl alcohol are coupled to pinoresinol. A so called dirigent protein leads to the exclusive formation of (+)-pinoresinol in *Forsythia intermedia* (Davin et al. 1997). Dirigent proteins or genes encoding them were also detected in other plant species leading to the assumption that the enantiomeric purity of the lignans is already determined by the coupling of the coniferyl alcohol molecules (Davin and Lewis 2003). (+)-Pinoresinol is reduced via (+)-lariciresinol to (-)-secoisolariciresinol by pinoresinol-lariciresinol reductase and subsequently oxidized to (-)-matairesinol (Dinkova-Kostova et al. 1996; Xia et al. 2001; Okunishi et al. 2004; von Heimendahl et al. 2005, Youn et al. 2005; Moinuddin et al. 2006). In contrast to the formation of (-)-matairesinol in *Forsythia* species the opposite lignan enantiomers can be found in other species, especially of the Thymeleaceae familiy (Umezawa 2003). Furthermore the studies on the enantiomeric composition of lignans in different plant species showed that in many cases enantiomeric purity is not reached before matairesinol opening the discussion on the general role of the dirigent proteins in lignan biosynthesis.

The steps from matairesinol to deoxypodophyllotoxin are most hypothetical since they were figured out by feeding of possible intermediates to *Anthriscus sylvestris* which accumulates mainly yatein (Sakakibara et al. 2003). In addition, the experiments of Seidel et al. (2002) led to the question whether yatein is really an intermediate in podophyllotoxin biosynthesis.

On the way to 6MPTOX the hydroxylation at position 6 of DOP by deoxypodophyllotoxin 6-hydroxylase (DOP6H) was proven to be catalyzed by a cytochrome P450 enzyme which was partially characterized in *L. flavum* and *L. nodiflorum* (Molog et al., 2001). *β*-Peltatin is converted to *β*-peltatin-A-methyl ether (PAM) by *β*-peltatin 6-*O*-methyltransferase (*β*P6OMT). This enzyme was characterized for the first time in *L. nodiflorum* (Kranz and Petersen, 2003).

In summary, it is obvious that the biosynthesis of PTOX and 6MPTOX is investigated in different systems. The hypothetical pathway is set up from results with plant species including such which do even not accumulate these lignans. Therefore, most steps have to be described as unknown even on a molecular level.

The biosynthesis of other lignans is almost not investigated up to now. But it is thought that the first steps from coniferyl alcohol to matairesinol are common in all pathways. Matairesinol is believed to be a central intermediate leading to all diverse lignan structures (Fig. 5).

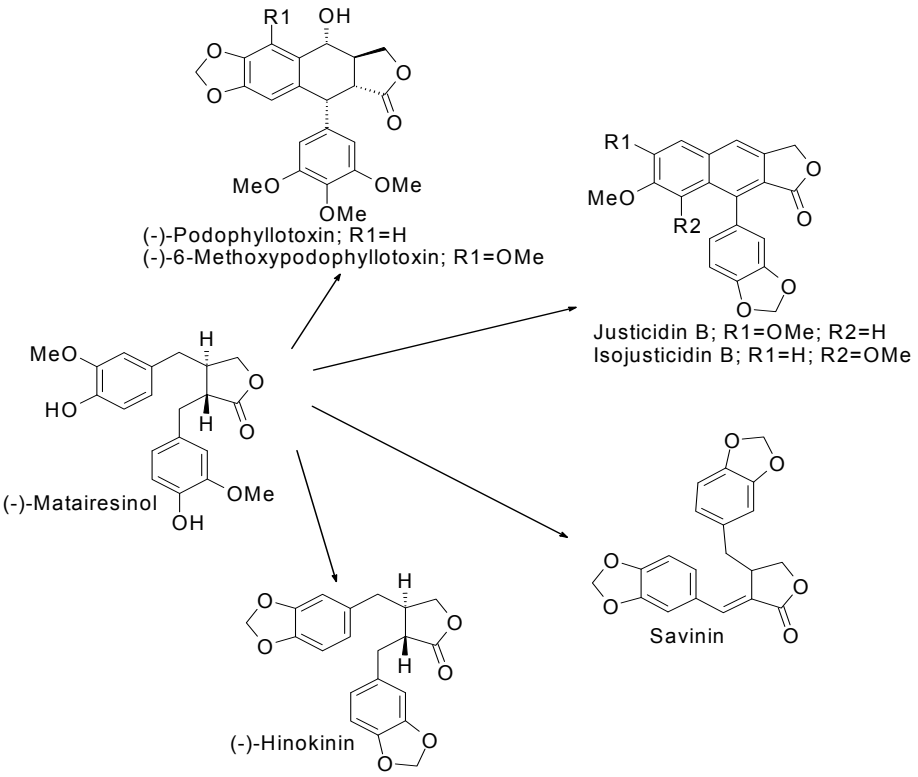


Fig. 5: Possibly from matairesinol derived lignans

3. Lignans in *in vitro* cultures of *Linum* species

Fuss E* (2003): Lignans in plant cell and organ cultures: An overview. Phytochemistry Reviews 2, 307-320

Van Fürden B, Humburg A, **Fuss E*** (2005): Influence of methyl jasmonate on podophyllotoxin and 6-methoxy-podophyllotoxin accumulation in cell suspension cultures of *Linum album*. Plant Cell Reports 24, 312-317

von Heimendahl CBI, Schäfer K, Eklund P, Sjöholm R, Schmidt TJ, **Fuss E*** (2005): Pinoresinol-lariciresinol reductases with different stereospecificity from *Linum album* and *Linum usitatissimum*. Phytochemistry 66, 1254-1263

Mohagheghzadeh A, Schmidt TJ, Bayindir Ü, **Fuss E**, Mehregan I, Alfermann AW (2006): Diarylbutyrolactone Lignans from *Linum corymbulosum in vitro* Cultures. Planta Medica 72, 1165-1167

Federolf K, Alfermann AW, **Fuss E*** (2007): Aryltetralin-tetralin formation in two different cell suspension cultures of *Linum album*: Deoxypodophyllotoxin 6-hydroxylase, a key-enzyme in the formation of 6-methoxypodophyllotoxin? (Phytochemistry, in press)

Hemmati S, Schmidt TJ, **Fuss E***: (+)-Pinoresinol/(-)-lariciresinol reductase from *Linum perenne* Himmelszelt involved in the biosynthesis of justicidin B (FEBS Letters, in press)

* corresponding author

3.1. Introduction

Lignans are important for humans due to the broad spectrum of functions for human health. The dibenzylbutyrolacton lignan HINO shows antiviral activity against the hepatitis B virus and anti-inflammatory activity (Huang et al. 2003; Da Silva et al. 2005). JusB, an arylnaphthalene lignan without any chiral center can attract interest, because of its fungicidal and antiprotozoal properties (Gertsch et al. 2003). It shows antiviral and anti-inflammatory activities as well as inhibition of platelet aggregation (MacRae et al. 1989; Chen et al. 1996; Rao et al. 2006). In addition, it is used as a lead compound for the design of antirheumatic drugs (Baba et al. 1996). Recently, its strong cytotoxicity on chronic myeloid and chronic lymphoid leukemia cell lines was shown (Vasilev et al. 2006). The aryltetralin lignan PTOX is used for the synthesis of anticancer drugs like etopophos®. Up to now the source for PTOX are roots and rhizomes of *Podophyllum hexandrum*, a plant endemic in the Himalayan region. As this plant species can not be cultivated, it became an endangered species in India (Imbert 1998; Giri and Narasu 2000). Strategies for the *in vitro* propagation of *Podophyllum peltatum* which accumulates PTOX beside other lignans in leaves were investigated (Moraes-Cerdeira et al. 1998). Chemical synthesis of PTOX is not economic on a commercial scale. Therefore, *in vitro* cultivation of plant material for the production of this useful natural compound was started several years ago (Petersen and Alfermann 2001).

Plant *in vitro* cultivation has several advantages over collecting plants from wild or cultivating them on fields (Alfermann et al. 2003). Metabolites like lignans can be produced under controlled and reproducible conditions, independent of geographical and climatic factors. Usually, it is not necessary to use herbicides or insecticides. Especially cell suspension cultures can show high growth rates combined with high accumulation of the desired metabolite in short time.

Different strategies can be followed to optimize the cultivation regime:

- Selection of a culture with high production of the desired compound
- Optimization of the medium for growth and accumulation
- Elicitation

There are a few examples where the *in vitro* cultivation of plants led to commercialisation of the process for the production of natural compounds. Already in 1982 Fujita et al. described a process for the production of the red antibiotic pigment shikonin in cell cultures of *Lithospermum erythrorhizon* which was followed by the company Mitsui Petrochemical Inc. Ltd. The cytotoxic paclitaxel (Taxol®) which is used in anticancer therapy is produced by the Phyton Ges. für Biotechnik mbH in cell cultures of *Taxus* species (Phyton 2002).

On the other hand, plant cell cultures are a good source to search for enzymes for secondary product formation (Zenk 1991). E. g., all enzymes for the production or rosmarinic acid were found in cell cultures of *Coleus blumei* (Petersen et al. 1995). In some cases the isolation of the enzymes has allowed the cloning of the corresponding cDNAs or genes (Kutchan et al. 1991). The modern molecular biological approaches like cDNA AFLP take advantage of plant cell cultures as well (Goossens et al. 2003; Rischer et al. 2006). The methods base on differential systems in which the accumulation of secondary metabolites is enhanced by e. g. elicitation to clone differentially expressed cDNAs which can have relevance to the

biosynthesis of the secondary compounds. By these approaches not only cDNAs for enzymes but also e. g. transcription factors can be cloned.
Until 2000 the main effort with respect to lignans was to establish cultures for the production of PTOX (reviewed in Fuss 2003). An optimization of the medium parameters in cultures of *P. hexandrum* for the production of PTOX gave only 0.075 % of the dry weight PTOX in 30 days of cultivation (Chattopadhay et al. 2003). In comparison, 3-4 % of the dry weight PTOX is found in roots and rhizomes of 4-5 year old plants of *P. hexandrum*. Cell cultures of *Linum album* showed the highest amount of PTOX ever observed in cell cultures (Smollny et al. 1998). The PTOX content reached about 0.3 % of the dry weight in only about two weeks of cultivation. Root-like cell cultures of *L. flavum* and cell cultures of *L. nodiflorum* reach with 0.7 % and 1.7 % of the dry weight the highest amounts of an arytetralin lignan in form of 6MPTOX in *in vitro* cultures ever observed. 6MPTOX shows comparable cytotoxic activity to PTOX and could therefore be an alternative to synthesize anticancer drugs like etopophos® (Van Uden et al. 1992).
Linum species and cultures thereof seem to be a good source for different types of lignans in general. Arylteralin lignans were found in cell cultures of *Linum album*, *L. flavum* and *L. nodiflorum*, all members of the section Syllinum in the genus *Linum*. Mohagheghzadeh found the arylnaphthalene type lignan justicidin B for the first time in *in vitro* cultures of a *Linum* species, *L. austriacum* which belongs to the *Linum* section Linum (Mohagheghzadeh et al. 2002).

3.2. Cell Cultures

We established cell cultures from several *Linum* species and investigated their lignan content. For this purpose we set up a couple of lignan extraction as well as analytical methods based on reversed phase and as chiral phases HPLC. HPLC-MS and NMR was done in close collaboration with Prof. Dr. T. J. Schmidt (University of Münster, formerly Pharmaceutical Biology, University of Düsseldorf) (e. g. Schmidt et al. 2006).
By screening several cell lines of *L. album* we identified lines with different lignan accumulation. Some lines accumulate only minor amounts of lignans (e. g. X4SF). Line PT is comparable to the line described by Smollny et al. (1998). It accumulates up to 5 mg/g dry weight PTOX as main lignan. In contrast, line 6M accumulates up to 7 mg/g dry weight 6MPTOX as main lignan. Further details are given in chapter 3.2.2. (Fuss 2003).
The diarylbutyrolactone hinokinin was detected for the first time in the genus Linum in *in vitro* cultures of *L. strictum* ssp. *corymbulosum* (Fig. 6) (Mohagheghzadeh et al. 2006). The fast growing cell suspension cultures - dry weight reaches the maximum of about 18 g/L within 10 days of cultivation - accumulate up to 0.36 mg/g dry weight hinokinin within 10 days of cultivation.

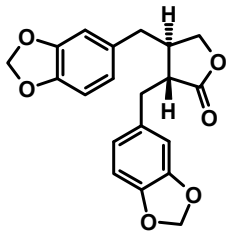


Fig. 6: (-)-Hinokinin

Mohagheghzadeh et al. (2002) established cell cultures of *L. austriacum*, a *Linum* species from section Linum which accumulates the arylnaphthalene lignan justicidin B. We could show that fast growing cell cultures from different *Linum perenne* varieties and subspecies which also belong to section Linum accumulate the same spectrum of lignans. Due to growth behaviour and lignan accumulation we decided to continue our work with cultures from *L. perenne* var. Himmelszelt. We could identify JusB as the main lignan together with several diphyllin glycosides (Fig. 7) (Hemmati et al, in press). To our knowledge this is the first report on the occurance of 7-oxygenated arylnaphthalene lignans and their derivatives in *Linum* species. The accumulation of justicidin B was with 23 mg/g dry weight 2-3 times higher than that observed in cell cultures of *L. austriacum* (Mohagheghzadeh et al. 2002).

justicidin B
(R=H)
diphyllin
(R=OH)
majidine
(R=O-apiose-xylose)
second diphylline glycoside
(R=O-hexose-pentose)

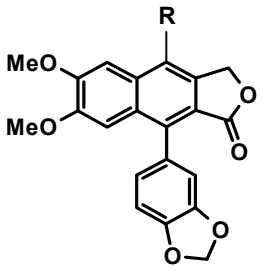


Fig. 7: Structures of justicidin B and diphyllin glycosides

3.2.1. Enzymes in cell cultures of Linum species (biochemistry and molecular biology)

PTOX and 6MPTOX biosynthesis in *Linum album*

In order to follow the biosynthesis of PTOX and 6MPTOX in a single plant system we use the cell cultures of *L. album* as a source to follow enzyme activities and clone cDNAs involved in the lignan pathway (Fig. 3, 4). Seidel at al. (2002) measured the activities of some enzymes from the general phenylpropanoid pathway throughout a cultivation period of a PTOX accumulating line: phenylalanin ammonia lyase (PAL), cinnamic acid 4-hydroxylase (C4H), cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD). An increase of the activities of these enzymes was observed concomitant with lignan accumulation which reached a maximum at day 10 of the cultivation period with about 3 mg/g dry weight PTOX. Almost no activities of the enzymes were measurable from day 10 on. We obtained comparable data with lines PT and 6M with one exception. CAD activities remained high until

the end of the cultivation period of 16 days (Federolf et al. in press). In addition, the activities of the deoxypodophyllotoxin 6-hydroxylase (DOP6H) and the *β*-peltatin 6-*O*-methyl-transferase (*β*P6OMT) involved in the biosynthesis of 6MPTOX were measured and biochemically characterised from *L. album* for the first time. Whereas *β*P6OMT activity was comparable in lines 6M and PT, DOP6H activity was almost not detected in line PT (for details see chapter 3.2.2. and Federolf et al. in press). All approaches to detect the 7-hydroxylation of deoxypodophyllotoxin (DOP) to PTOX or PAM to 6MPTOX in assays for cytochrome P450 monooxygenase, dioxygenases and peroxidases which are candidate enzyme classes for these hydroxylations failed up to now. Therefore, we conducted biotransformation experiments using cell lines PT and 6M incubated with DOP together with inhibitors of cytochrome P450 monooxygenases and dioxygenases in order to shed some light on the nature of the enzymes involved in 7-hydroxylation (Fig. 8) (Federolf et al. in press). Administrated DOP was converted to PTOX in line PT (data not shown) and to PAM in line 6M. DOP incubated with *Podophyllum hexandrum* and *L. flavum* cell cultures is converted to PTOX and 6MPTOX, respectively (Van Uden et al. 1995). Accumulation of PAM instead of 6MPTOX in our case might occur because PAM7H is already saturated with the PAM in the cells without feeding of DOP or the time point of harvest was too early to see the conversion of the PAM to 6MPTOX. Inhibitor concentrations up to 10^{-4} M had no influence on PTOX accumulation in line PT. The formation of PAM via *β*-peltatin initiated by the activity of DOP6H was also not influenced by addition of the dioxygenase inhibitors in the same concentration. But, PAM levels were significantly reduced after addition of 10^{-4} M of the cytochrome P450 monooxygenase inhibitors showing that cytochrome P450 monooxygenases like the DOP6H can be inhibited *in vivo* by the inhibitors chosen. On the other hand, the PTOX levels were increased after addition of the cytochrome P450 monooxygenase inhibitors. From that one can take two conclusions. First, DOP7H is not a cytochrome P450 monooxygenase. Second, the reaction from DOP to PTOX which is presumed to be catalyzed by DOP7H might be catalyzed by PAM7H as byproduct since PTOX is only accumulated in higher amounts if DOP6H is missing and therefore the right substrate for the PAM7H, the PAM, is not delivered.

We established cDNA libraries of lines PT and 6M from the cultivation days four to seven in order to clone cDNAs involved in the biosynthesis of PTOX and 6MPTOX.

We have cloned two cDNAs encoding PAL (A. Schwelm), and one cDNA encoding C4H (A. Korczak), CCR (J. Windhövel), CAD (A. Schwelm, L. Neumetzler) and PLR each. The proteins except the C4H were heterologously expressed in *E. coli* and biochemically characterised (data not shown).

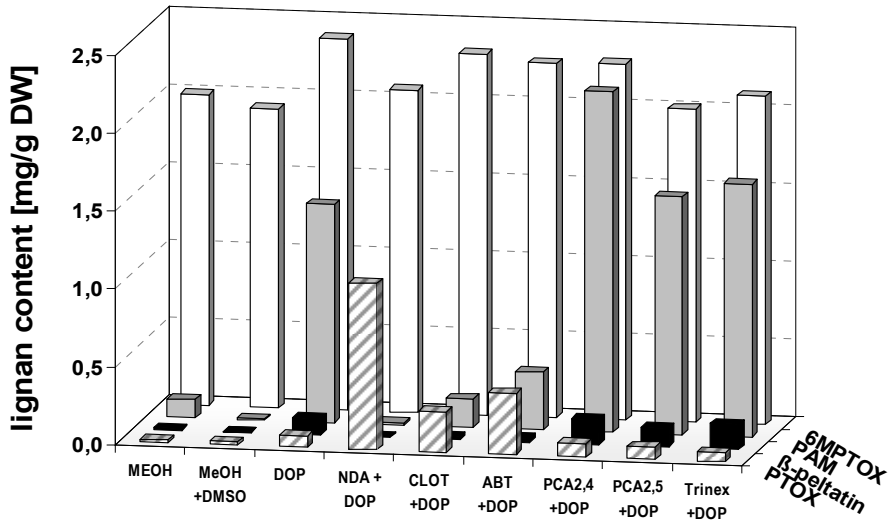


Fig. 8: Influence of NDA (in DMSO), ABT (in MeOH), CLOT (in MeOH), 2,4-PCA (in DMSO), 2,5-PCA (in DMSO) or trinex (in MeOH) on the content of *β*-peltatin (**2**), PAM (**3**), 6MPTOX (**4**) and PTOX (**5**) in cells of line 6M. The inhibitors were added at a final concentration of 10^{-4} M at day 4. 2.5 x 10^{-4} M DOP (**1**) (in MeOH) was administered at day 5. The lignan content was determined after harvest at day 7
[Inhibitors for cytochrome P450 monooxygenases: ABT, 1-aminobenzotriazole; CLOT = clotrimazole and NDA = tetcyclacis; inhibitors for dioxygenases: 2,4-PCA = 2,4-pyridinedicarboxylic acid; 2,5-PCA = 2,5-pyridinedicarboxylic acid and trinex = trinexapac-ethyl (Pestanal®)]

Hinokinin biosynthesis in *Linum corymbulosum*

The only report on biosynthesis of hinokinin (HINO) was reported by Takaku et al. in 2001. They isolated eleven lignans from the heartwood of *Chamaecyparis obtusa*, namely xanthoxylol, 7-oxohinokinin, savinin, dihydrosesamin, isoactifolin, (-)-sesamin, piperitol, HINO, (-)-pluviatolide, (-)-haplomyrfolin and (-)-matairesinol. Based on the chemical structures of these lignans they suggested possible biosynthetic pathways leading to HINO (Fig. 9). In both pathways (+)-pinoresinol (PINO) is the central intermediate. If first a two-step-reduction by a pinoresinol-lariciresinol reductase (PLR) happens, (-)-secoisolariciresinol will be formed, then a secoisolariciresinol dehydrogenase will form (-)-matairesinol, from which HINO is synthesized by the formation of two methylene dioxy bridges. If the two methylene dioxy bridges are formed directly on PINO by piperitol-sesamin synthase (PSS) to give (+)-sesamin, a two-step-reduction similar to the reductions catalysed by PLR gives (-)-dihydrocubebin. Then the last reaction to form HINO is a dehydrogenation reaction like the one catalysed by the SDH.
In order to get insight into which pathway is really involved in HINO biosynthesis, we tried to clone cDNAs encoding proteins possibly involved in all described reaction steps.

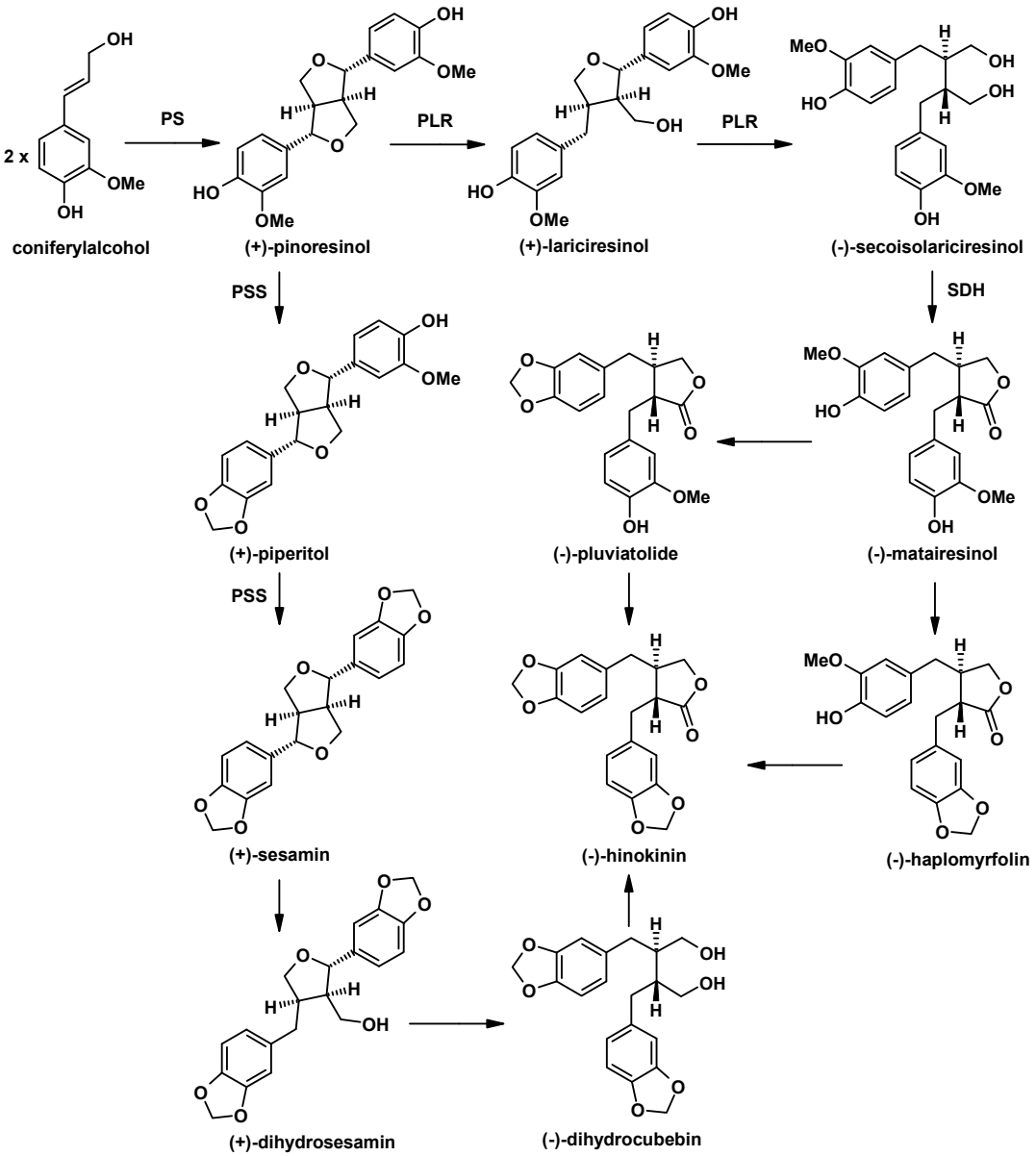


Fig. 9: Hypothetical biosynthetic pathways leading to (-)-hinokinin (modified according to Takaku et al. 2001)

Up to now, we amplified a part of a cDNA ("G") encoding a protein with high similarity to phenylcoumaran benzylic ether reductases (PCBERs) and isoflavone reductases (IFRs) which belong to the same family with PLRs. We established a cDNA library with RNA collected from a cell culture of *L. corymbulosum* from days 3 to 7 of the cultivation period. Until now four clones containing full length sequences with highest similarities to PLRs, PCBERs and IFRs were obtained. The proteins were heterologously expressed as 6His fusion proteins in *E. coli* and purified to homogeneity. Dehydrodiconiferyl alcohol and racemic PINO were used as substrates for PCBER and PLR assays, respectively. Three proteins have PCBER activity. In addition, one protein with highest similarity to PLRs has PLR activity. In order to determine the enantiospecificity of this PLR we used racemic PINO as substrate in assays which were stopped after different duration (Fig. 10). The PLR from *L. corymbulosum* is specific for the conversion of (+)-PINO to (-)-SECO via (+)-LARI. This enantiospecificity fits into the pathway leading to (-)-HINO giving a first hint, that the pathway

via matairesinol and not sesamin is the right pathway leading to HINO. RNAi experiments to knockdown the expression of this PLR are ongoing as proof for this assumption.

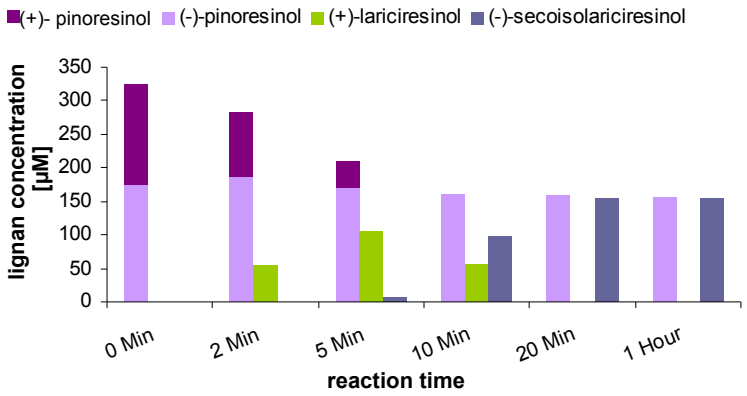


Fig. 10: Conversion of (+)- and (-)-pinoresinol from a racemic mixture with a purified heterologously expressed PLR from *L. corymbulosum* (One example of three independent determinations is shown.)

Justicidin B and diphyllin biosynthesis in *L. perenne*

Nothing is known about the biosynthesis of the arylnaphthalene type lignans justicidin B (JusB) and diphyllin (Diph). But, one can presume that the biosynthesis starts with the dimerisation of coniferyl alcohol to pinoresinol which is further converted to matairesinol (Fig. 11). Several unknown steps can lead to the biosynthesis of JusB which can be hydroxylated at position 7 to give Diph. Diph can be glycosylated to several Diph-glycosides.

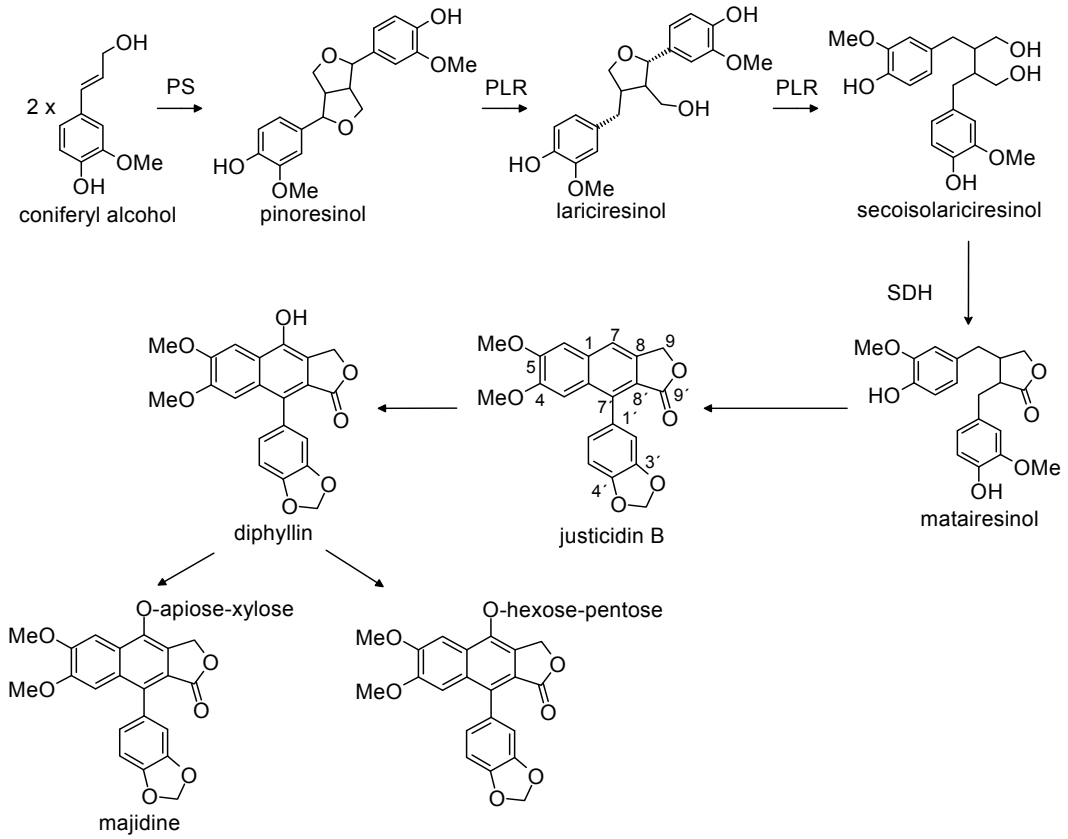
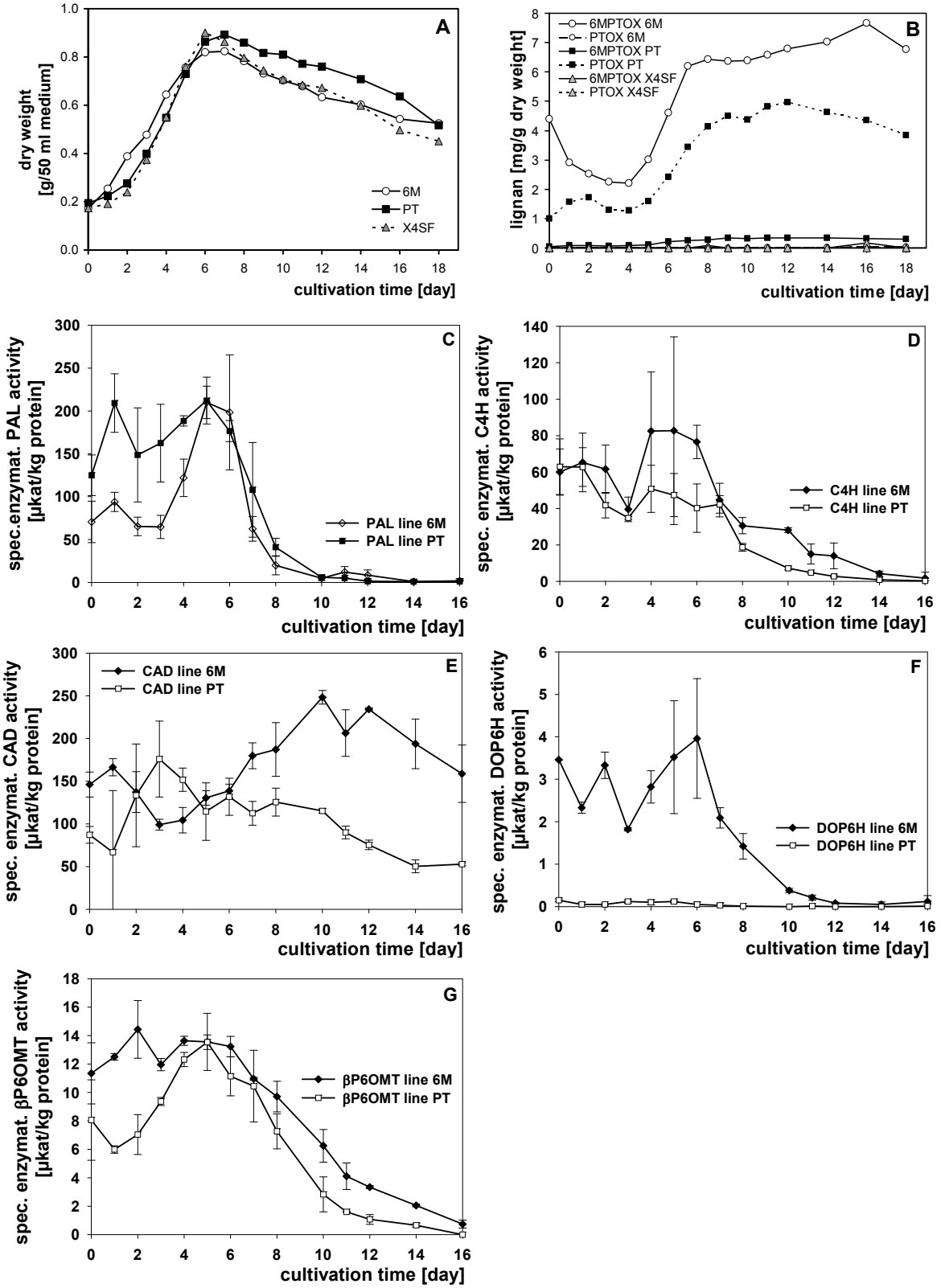


Fig. 11: Hypothetical biosynthetic pathway leading to justicidin B and diphyllin glycosides

We could isolate a cDNA encoding a PLR by a RT-PCR approach (Hemmati et al. in press). The heterologously expressed protein prefers (+)-PINO, but (-)-LARI. Therefore this PLR changes its enantio-preference from lignans with R,R configuration at C-atoms 8,8' to ones with S,S configuration. RNAi experiments proofed its contribution in the biosynthesis of JusB and Diph-glycosides.
Recently, we could show the hydroxylation of JusB to diphyllin in cell free membrane fractions of *L. perenne* Himmelszelt cell cultures. The reaction can be inhibited by cytochrome c which indicates that the JusB 7-hydroxylase is a cytochrome P450 monooxygenase. Further biochemical characterisation of the enzyme is ongoing.

3.2.2. Differential accumulation of lignans due to genotypic variations

The variability of the accumulation of secondary compounds in different cell lines of the same plant species is a phenomenon often observed (Zenk et al. 1977; Zenk 1978). This effect was summarized under the name somaclonal variation (Larkin and Scowcroft 1981). The basis can be genetically, epigenetically or physiologically. We observed all types of variations with cell cultures of *Linum album*.
Numerous cell cultures from individual *L. album* seedlings were established. Phytochemical analysis revealed that from 12 lines investigated, two lines show almost no accumulation of PTOX or 6MPTOX, two lines accumulate mainly PTOX and eight lines accumulate 6MPTOX as main lignan. One reason for that can be the probably different genotype of the seedlings from which the cultures were initiated. The seeds were collected from different wild growing plants from varying places in Iran indicating different genotypes. On the other hand we observed loss of the capability for or changes in lignan accumulation in the cultures under investigation from time to time. Epigenetic changes may be responsible. Some of the cultures were compared with respect to lignan accumulation, growth and biosynthesis of lignans (Fig. 12) (Fuss 2003, Federolf et al. in press, unpublished data).
Whereas line 6M is an example for a culture with accumulation of 6MPTOX, line PT accumulates mainly PTOX. The total levels of both lignans are comparable in these lines. Neither the growth parameters nor the specific activities of some enzymes involved in the biosynthesis of lignans, PAL, C4H, CAD and *β*P6OMT, showed large differences between the lignan accumulating lines 6M and PT. The most striking difference was the activity of DOP6H, which was almost only detectable in line 6M. In all lines investigated so far, DOP6H activity was only found if considerable amounts of 6MPTOX were accumulated. Therefore, the presence of DOP6H might be a key to channel the precursor deoxypodophyllotoxin for formation of PTOX or 6MPTOX.



(Fig. will be continued on the next page)

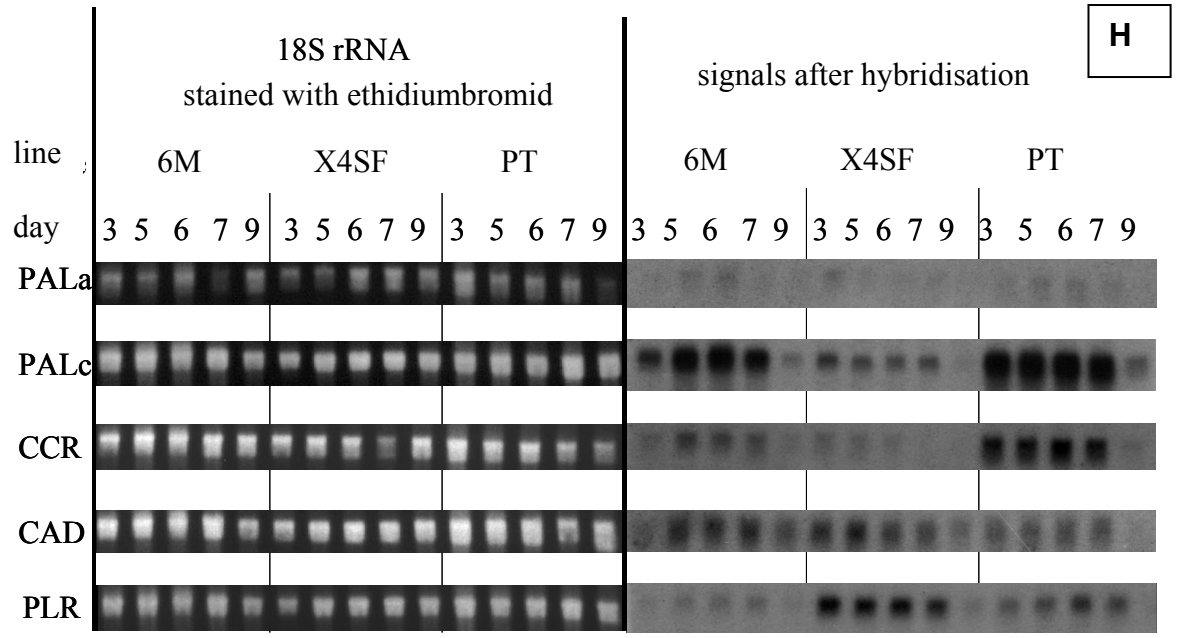


Fig. 12: Characterisation of *L. album* cell suspension cultures 6M (=1-1-4), PT (=HermA) and X4SF
Time course of **A:** PTOX (**5**) and 6MPTOX (**4**) accumulation; **B:** dry weight (DW); **C:** Phenlylalanine ammonia lyase (PAL) activity; **D:** Cinnamic acid 4-hydroxylase (C4H) activity; **E:** Coniferyl alcohol dehydrogenase (CAD) activity; **F:** Deoxypodophyllotoxin-6-hydroxylase (DOP6H) activity; **G:** *β*-peltatin 6-*O*-methyltransferase (*β*P6OMT) activity of line 6M and PT over a cultivation period of 16 days. **H**: Northern analysis (The characterisation is done in duplicate; each measurement of enzyme activities within one characterisation is carried out in triplicate.) (Federolf et al. in press and unpublished data)

Line X4SF shows almost no accumulation of lignans. It resulted from a line with mainly PTOX accumulation probably by somaclonal variation. To investigate this effect further we compared the mRNA levels for some enzymes involved in lignan biosynthesis with the levels in lines 6M and PT over a cultivation period of 9 days (Fig. 12, H). Whereas PALa and CAD mRNA levels were almost not distinguishable in the three lines, PALc and CCR mRNA accumulation was lower and PLR accumulation was higher than in the lines PT and 6M. This gives hints to multiple effects on lignan accumulation in line X4SF. Since PAL is the entrance enzyme to the lignan biosynthetic pathway already the reduction in PALc mRNA levels would be sufficient to explain the low lignan levels in line X4SF. PAL activity was also much lower in this line in comparison to lines 6M and PT, too (data not shown). The reduction in PLR mRNA levels can be explained by a feed back mechanism due to the low levels of the end products PTOX and/or 6MPTOX.

3.2.3. Elicitation

An elicitor is a physical, chemical or biological stimulus that triggers defence responses in plants. A typical response of plants against enemies is the new formation or enhancement of secondary metabolite accumulation. These secondary metabolites can be of different structural types, but are specific for single plant species or often taxonomic groups of plants. They are referred to phytoalexines. The signal transduction in elicitation involves one or more of a trio of regulators – jasmonate, ethylene and salicylic acid. Extra cellular elicitors as well as methyl jasmonate (MeJas) have been used to increase the formation of natural products in plant cell cultures (Messner and Boll 1993; Szabo et al. 1999; Schmitt and Petersen 2002; Goossens et al. 2003). Only in one case the enhancement of PTOX accumulation up to 1.4 µg/g dry weight by a chito-oligosaccharide was described for cell cultures of *Juniperus chinensis* (Muranaka et al. 1998).
We have screened several of our suspension cultures from *L. album* for elicitation by 100 µM MeJas. Some examples are given in Fig. 13. Most lines did not show a significant increase in level of PTOX or related lignans after addition of MeJas in comparison to a solvent control (Fuss 2003, Van Fürden et al. 2005). If there was a good response, e. g. about 10-fold increase in PTOX content in line X4SF, the lines usually accumulate only minor amounts of lignans without MeJas. The growth of the cultures was not influenced by ethanol (0.025 %), the solvent used for MeJas. 100 µM MeJas leads only to a slight decrease of the dry and fresh weight accumulation (Van Fürden et al. 2005).

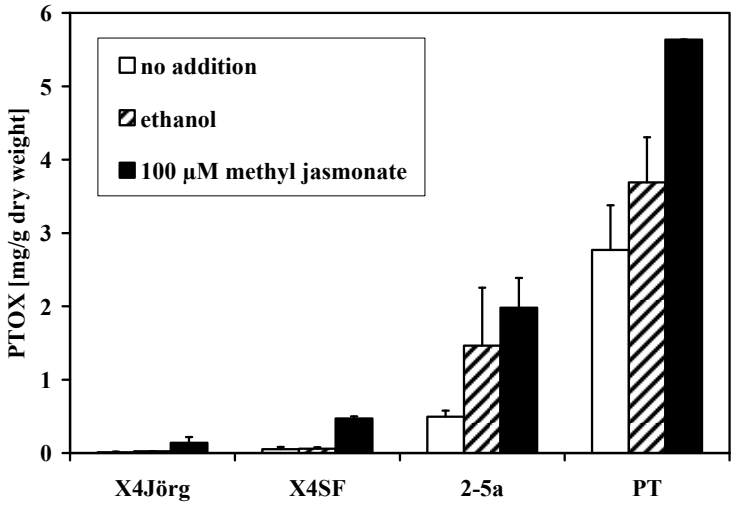


Fig. 13: Effect of 100 µM methyl jasmonate in ethanol on PTOX accumulation in different cell cultures of *L. album*

Line X4SF showed the highest increase in PTOX accumulation after elicitation with 100 µM MeJas from the lines investigated. Therefore, this line was chosen for further investigations of the MeJas effect on lignan biosynthesis (Fig. 14).

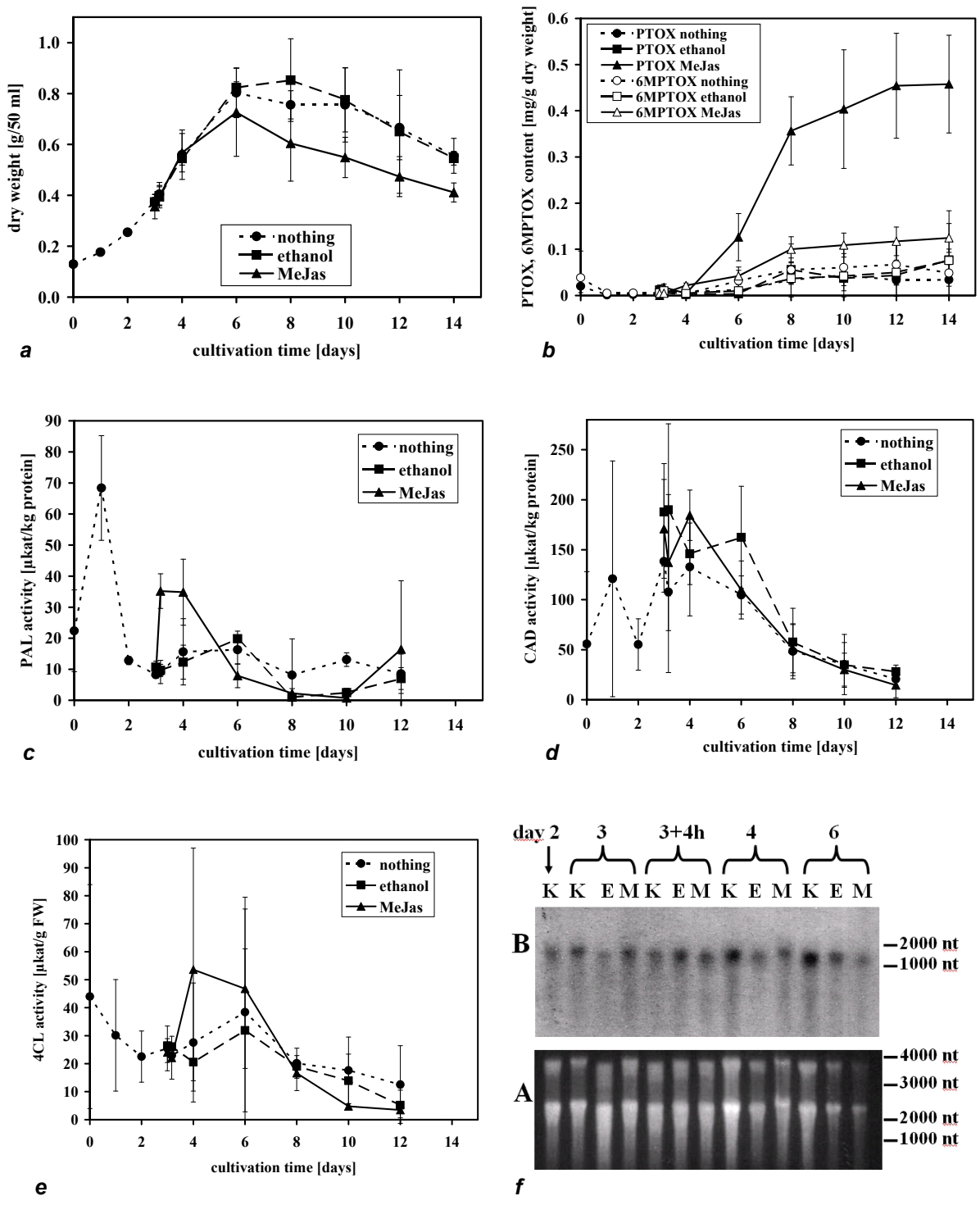
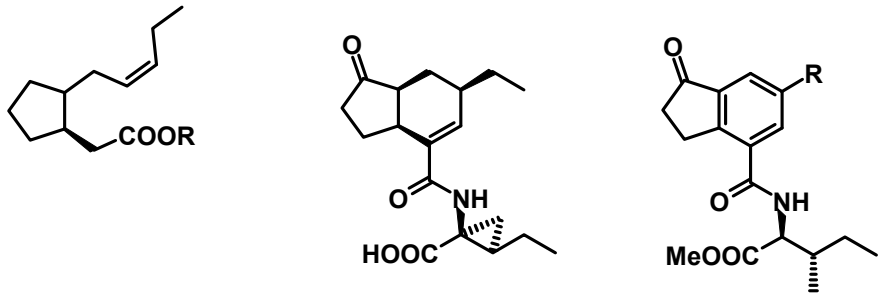


Fig. 14: Effect of 100 µM MeJas added to cell culture X4SF of *L. album* on day 3 on:
a: dry weight accumulation, b: PTOX and 6MPTOX content, c: phenylalanine ammonia-lyase (PAL) activity, d: cinnamyl alcohol dehydrogenase (CAD) activity, e: 4-coumarate ligase (4CL) activity f: PLR mRNA accumulation (K: nothing, E: ethanol, M: MeJas, A: gel stained with ethidium bromide, B: after hybridisation with the 3'-UTR as probe) (Van Fürden et al. 2005 and unpublished results)

100 µM MeJas as well as the solvent ethanol were added at day 3 of the cultivation period on culture X4SF and the following parameters measured during a cultivation period of 14 days: dry weight to follow the growth and the content of PTOX and 6MPTOX as well as activities or mRNA accumulation of enzymes involved in the lignan biosynthesis (van Fürden et al. 2005 and unpublished results). Ethanol has no significant influence on any of the parameters investigated. The addition of MeJas led to a slight decrease in dry weight. The PTOX content increased one day after addition of MeJas from 0.01 mg/g dry weight to 0.45 mg/g dry weight at day 12, and then remaining constant until the end of the cultivation period. 6MPTOX content did not show such a high increase. It started to increase already 4 hours after addition of MeJas from 0.01 mg/g dry weight to 0.10 mg/g dry weight at day 8, followed by a slight increase to 0.13 mg/g dry weight until the end of the cultivation period. Whereas cinnamoyl alcohol dehydrodgenase activity and pinoresinol-lariciresinol reductase mRNA levels were not influenced by MeJas, the activities of the phenylalanine ammonia lyase (PAL) and the 4-coumarate ligase (4CL) were increased in comparison to the controls 4 hours and 1 day or only one day after addition of MeJas, respectively. Since the increase in lignan levels is coupled with an increase of PAL and 4CL activity, we conclude that at least these steps are rate limiting in unelicited cells, whereas CAD and PLR seems to be present in sufficient amounts for biosynthesis of at least 0.45 mg/g dry weight PTOX and 0.13 mg g dry weight 6MPTOX.

Unfortunately, the culture X4SF lost its capability for enhancement of lignan accumulation by elicitation with MeJas. Therefore, we had to screen our cell lines again and checked new substances for their influence on PTOX biosynthesis. After Schüler at al. described the in most cases higher effectiveness of derivatives of the bacterial phytotoxin coronatine in 2004 (Fig. 15), Berim et al. (2005) tested coronalone (CR) and indanoyl-isoleucin (II) in comparison to MeJas in cultures of *Linum nodiflorum*. Already small amounts of CR or II led to an increase in 6MPTOX formation accompanied by the accumulation of a new lignan: 5'-demethoxy-6MPTOX. Maximum enhancement up to 2.5 % of the dry weight 6MPTOX was reached with 50 µM CR or II.



R=H jasmonic acid
R=CH_3 methyl jasmonate

coronatine

R=H indanoyl-isoleucine (II)
R=CH_2CH_3 coronalon (CR)

Fig. 15: Structures of jasmonate-related compounds and mimics

We tested whether these new elicitors are also effective in cell cultures of other *Linum* species. Whereas MeJas administered in concentrations up to 300 µM had no influence on the accumulation in cell cultures of *L. strictum* ssp. *corymbulosum*, already 50 µM Cr or II led to an increase on hinokinin content from 40 µg/g dry weight in control cells up to 150-200 µg/g dry weight in elicitor-treated cells.

From the cell cultures of *L. album* investigated line PT (synonym 2-5aH) showed the best response with respect to PTOX as well as 6MPTOX accumulation. Therefore, this culture was chosen to investigate the elicitor effects in more detail (Fig. 16) (H.G. Fuhrmann).

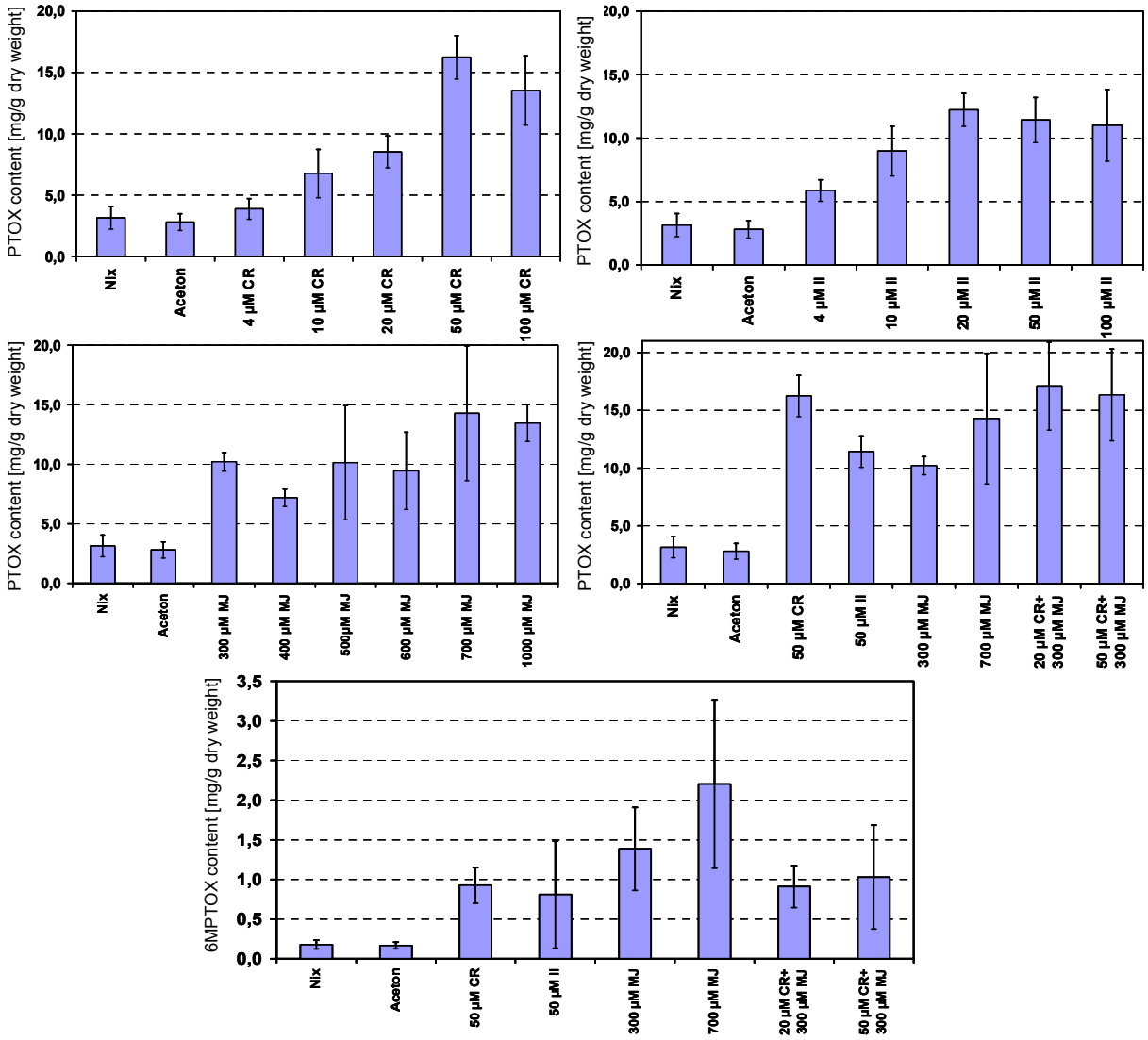


Fig. 16: Effect of different concentrations of MeJas (MJ) CR and II on the accumulation of PTOX and 6MPTOX in cell line PT of *Linum album*. Compounds were administered at day 3 and the cell cultures harvested at day 12 of the cultivation period. Values represent mean +/- SD (n=4) (H. G. Fuhrmann).

CR and II enhance PTOX accumulation already at concentrations as low as 4 µM. Van Fürden et al. (2005) could enhance the PTOX content up to app. 7.7 mg/g dry weight by addition of 100 µM MeJas. Only one tenth, 10 µM, of CR or II gave the same result. Administration of 50 µM CR gave highest PTOX contents with app. 17 mg/g dry weight (app. 250 mg/l). This is the highest amount of PTOX ever observed in cell cultures up to now. Highest 6MPTOX contents of app. 2.2 mg/g dry weight were reached after addition of 700 µM MeJas. Combinations of MeJas and CR in suboptimal concentrations lead to an additive effect on PTOX accumulation but not 6MPTOX accumulation indicating the preference of PTOX accumulation in line PT, which is already described by Federolf et al. (in press). The influence of MeJas and CR on activities of the enzymes in the biosynthesis of PTOX and 6MPTOX is currently under investigation.

3.4. Conclusion

In vitro cultures of *Linum* species are a good source for different types of lignan structures. The same types of lignans as they occur in the plant are accumulated in these cultures in most cases. Therefore, these cultures are a good alternative to the plants itself which are not always easily available.

We have cell cultures and hairy roots in hand which accumulate three different subtypes of lignans: *L. corymbulosum* the dibenzylbutyrolacton lignan hinokinin, *L. perenne* var. Himmelszelt the arylnaphthalene lignans justicidin B and diphyllin glycosides and *L. album* the aryltertralin lignans podophyllotoxin and 6-methoxypodophyllotoxin. We have already cloned some cDNAs probably involved in the pathways leading to the different lignan structures.

The most interesting pathway is the one leading to PTOX and 6MPTOX due to the strong use of PTOX for human health (Imbert 1998). It is under investigation since about the last 30 years. But our knowledge is still very limited. The reasons could have been the absence of valuable systems. Furthermore, the enzymes which should be involved belong to the most difficult ones for biochemical as well as molecular biological investigations due to weak sequence similarities and/or low abundance and/or difficulties within the extraction procedures, namely cytochrome P450 monooxygenases, dioxygenases, peroxidases and *O*-methyltransferases (Fig. 17).

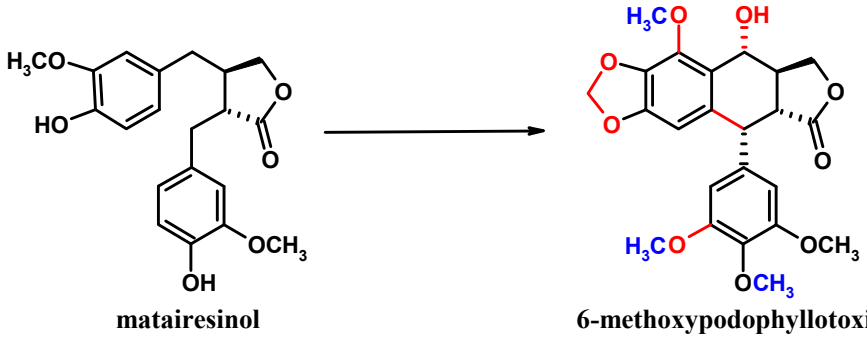


Fig. 17: Changes in the molecular structure between matairesinol and 6-methoxypodophyllotoxin which could be catalysed by cytochrome P450 monooxygenasen, dioxygenases or peroxidases (red) and *O*-methyltransferases (blue)

Therefore, new molecular biological approaches based on differential gene expression to clone genes should be used (e.g. cDNA-AFLP, Goosens et al. 2003). These methods don't have the prerequisite of knowledge of the type of gene looked for. We have established two systems with *L. album* cell cultures. The first system is based on the differential lignan accumulation in the different cell lines of *L. album*. The second one is based on the elicitation to enhance PTOX accumulation. We could show that the enzyme activities and/or mRNA accumulation levels of a few already known enzyme/genes probably involved in PTOX/6MPTOX biosynthesis were distinguishable in cultures with different lignan accumulation in both cases. This indicates that our systems will be useful as basis for modern molecular biological techniques.

4. Genetic Manipulation of the lignan biosynthesis in *Linum* species

Fuss E (2003)*: Lignans in plant cell and organ cultures: An overview. Phytochemistry Reviews 2, 307-320

Mohagheghzadeh A, Schmidt TJ, Bayindir Ü, Fuss E, Mehregan I, Alfermann AW (2006): Diarylbutyrolactone Lignans from *Linum corymbulosum in vitro* Cultures. Planta Medica 72, 1165-1167

Hemmati S, Schmidt TJ, Fuss E*: (+)-Pinoresinol/(-)-lariciresinol reductase from *Linum perenne* Himmelszelt involved in the biosynthesis of justicidin B (FEBS Letters, in press)

Windhövel J, S. Steegmüller S, Immeln D, Mohagheghzadeh A, Alfermann AW, Fuss E*: *Agrobacterium rhizogenes*-mediated manipulation of the lignan biosynthesis in *Linum album* and *Linum persicum* (will be submitted soon)

* corresponding author

4.1. Introduction

Secondary plant metabolites like the lignans have multiple functions throughout the plant life cycle as mediators in the interaction of the plant with its environment. In addition, several plant secondary metabolites are used for the production of medicines, dyes, flavours, insecticides and fragrances. Therefore, plant secondary metabolism is an interesting target for plant breeding. Molecular breeding by applying genetic engineering is a promising approach which was followed with respect to secondary metabolism within the last 15 years (Verpoorte and Alfermann 2000; Verpoorte and Memelink 2002). Secondary metabolic pathways are genetically manipulated to increase, newly introduce or decrease the quantity of a certain compound or group of compounds. To increase the production either one or a few rate-limiting steps can be overcome by increasing their expression or by the change of

the expression of regulatory genes which control genes responsible in several steps of the biosynthetic pathway to the secondary compound. The decrease of the accumulation of an unwanted compound or group of compounds can be achieved by several approaches. The most prominent are the reduction of the levels of corresponding mRNA(s) or inactivation of an enzyme by overexpression of antibodies against it. The effectiveness in reducing mRNA levels in plants has been highly improved since the discovery of RNAi (Watson et al. 2005).
Unfortunately, most of the biosynthetic pathways for secondary plant metabolites are still at least partially hypothetical. Therefore, very few genes responsible for secondary compound formation are known and can be targets for genetic manipulation. The biosynthesis of the monolignols, the precursors for lignin, lignans etc., is one of the most studied pathways of plant secondary product biosynthesis. Genes for all steps were cloned and used to genetically manipulate this pathway (Dixon and Reddy 2003). But, the influence of the alterations in the expression levels of genes involved in that pathway are not investigated with respect to lignan biosynthesis up to now. Only a few genes probably involved in the steps from the monolignols to lignans were cloned so far. In addition, the plant species from which the genes are available are not the model plants which can be easily genetically manipulated. That could explain why no experiments for the genetic engineering of lignan biosynthesis are described up to now.

4.2. Agrobacterium rhizogenes-mediated transformation of Linum species

Many plant secondary metabolites are accumulated in roots. The highest content of the medicinal important lignan PTOX is found in roots and rhizomes of *Podophyllum hexandrum* (Imbert 1998).
The infection of plants with the soil bacterium *Agrobacterium rhizogenes* causes tumour formation by introducing a set of genes which control auxin and cytokinin biosynthesis. The hormonal imbalance causes the formation of proliferating roots, called hairy roots. Fast and hormone-independent growth and high genetic stability make hairy root cultures superior in comparison to cell cultures. Hairy roots are known to produce the same or even higher amounts of the metabolites found in normal roots. Therefore, much effort was made to use hairy roots for the production of secondary metabolites during the last 20 years (Guillon et al. 2006).
When we started our work, only a few examples of the production of lignans in hairy roots were available. Hairy roots from *Sesamum indicum* accumulate sesamin and sesamolin (Khanna and Jain 1973; Mimura et al. 1987; Ogasawara et al. 1998). Hairy roots of *Linum flavum* produce 1.5 to 3.5 % of 6MPTOX on a dry weight basis, which is 2 to 5 times higher than the 6MPTOX content in untransformed roots and 5 to 12 times higher than in *L. flavum* cell suspensions (Oostdam et al. 1993). Whereas cell cultures of *Linum austriacum* accumulate 6.7 mg/g dry weight justicidin B, hairy roots of the same species can accumulate 16.9 mg/g dry weight justicidin B (Mohagheghzadeh et al. 2002).
Since one part of our research focuses on the biosynthesis of PTOX and 6MPTOX in *L. album* our first goal was to establish hairy roots from this plant species and the closely

related *L. persicum*. Both species are endemic in Iran. We initiated shoot cultures of these species in Murashige Skoog medium without phytohormones. Our first attempts to transform these cultures with different strains of *Agrobacterium rhizogenes* (ATCC 15834, LBA 9402, TR 105, and R1600) failed. Only in one case we could establish a hairy root culture from *L. album* infected with the *A. rhizogenes* strain TR 105 (line 3-4) which accumulates 28 mg/g DW 6MPTOX within 14 days accompanied by trace amounts of PTOX. Therefore, we infected the hypocotyls of *in vitro* germinated seedlings. The germination rate was very variable depending on the seed source and age. Usually we could not observe more than 15 % germination. Most of the seedlings were not reacting to the Agrobacteria, obviously independent of the agrobacterial strain used. If one seedling could be infected the hairy roots either appeared directly at the hypocotyl or callus formation was observed first. On most responsive seedlings (app. 40 % of the germinated seeds) hairy root formation occurred at several independent positions. Probably the different responsiveness of the seedlings is due to their different genotypes since the seeds were collected from wild grown plants. Finally we could establish 25 hairy root cultures from 3 *L. album* seedlings which accumulate 35-45 mg/g DW 6MPTOX, 0.03-2.26 mg/g DW *β*-peltatin and 0-0.25 mg/g DW PTOX and α-peltatin (not quantified). 13 lines were established from 5 *L. persicum* seedlings accumulating 40-58 mg/g DW 6MPTOX, 0.29-2.28 mg/g DW *β*-peltatin and 0.20-0.25 mg/g DW PTOX. These are the highest 6MPTOX contents ever observed in *in vitro* cultures (Fuss, 2003). Roots of *L. persicum* plants show a comparable pattern of lignan accumulation (Mohagheghzadeh et al., 2003). They contain 6MPTOX as main lignan together with α-peltatin and trace amounts of PTOX. Hairy roots initiated from leaves of *Linum flavum* produce 14-35 mg/g DW 6MPTOX and trace amounts of β-peltatin, α-peltatin and PTOX, therefore showing less 6MPTOX and a higher variability between the different lines (Oostdam et al., 1993). A low variability between the different lines is desirable to have a stable baseline for genetic manipulations of the 6MPTOX biosynthesis. In contrast to our hairy root lines, cell suspension cultures of *L. album* show a very high variability of lignan accumulation (Fuss, 2003). Some lines accumulate only trace amounts of lignans whereas others accumulate up to 5 mg/g DW PTOX or 8 mg/g DW 6MPTOX as main lignans.

The transformation status of the hairy roots was analysed by PCR (Fig. 18). A specific set of primers was used to amplify a piece of app. 540 bp of the gene *rolC* responsible for the hairy root genotype. To be able to distinguish the integration of the *rolC* gene into the plant genome and to rule out the possibility of residual growth of *A. rhizogenes* in the hairy roots a second PCR with primers specific for the *virC* gene generating a fragment of app. 730 bp was performed. After several subcultivation cycles in medium containing timentin® each hairy root line was free from *A. rhizogenes* and was confirmed to contain the *rolC* gene.

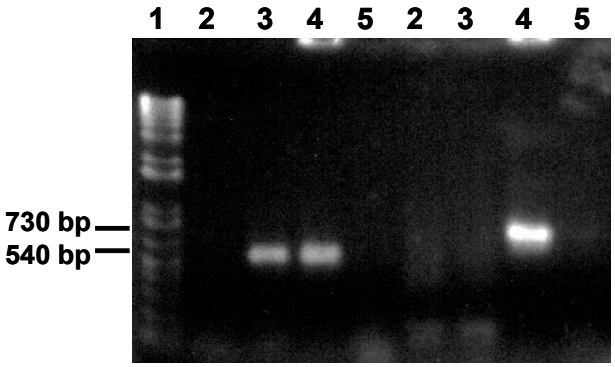


Fig. 18: Proof of transformation by PCR amplification of fragments from *rolC* and *virC*
1: 1 kb plus ladder (Invitrogen)
2: gDNA from untransformed tissue (cell suspension culture from *Linum album*)
3: gDNA from transformed hairy roots from *Linum album* (line 3-4)
4: positive control (TR105)
5: negative control (water)

The *L. album* hairy root line 3-4 was grown in McCown with 2 % sugar over a cultivation period of 36 days (Fig. 19). The highest dry weight was reached at day 14 (app. 11 g/l). At the same time the sugar content in the medium was dropped to almost zero. The conductivity as indicator of the ion content in the medium reached also its minimum of app. 1.25 mS. Since the conductivity never decreased further it was thought that the sugar was limiting the growth. Experiments with 3 % instead of 2 % sugar as starting point did not alter the time course of conductivity and did not led to a significant increase in dry weight accumulation (data not shown). The line 3-4 shows a higher growth rate (5 days doubling time) than the cultures of *L. flavum* (9-28 days doubling time, Oostdam et al. 1993). The accumulation of 6MPTOX paralleled the growth course. The 6MPTOX content in the cells reached the maximum at day 18 (app. 34 mg/100 ml, 32 mg/g DW) and remained stable until the end of the cultivation period.

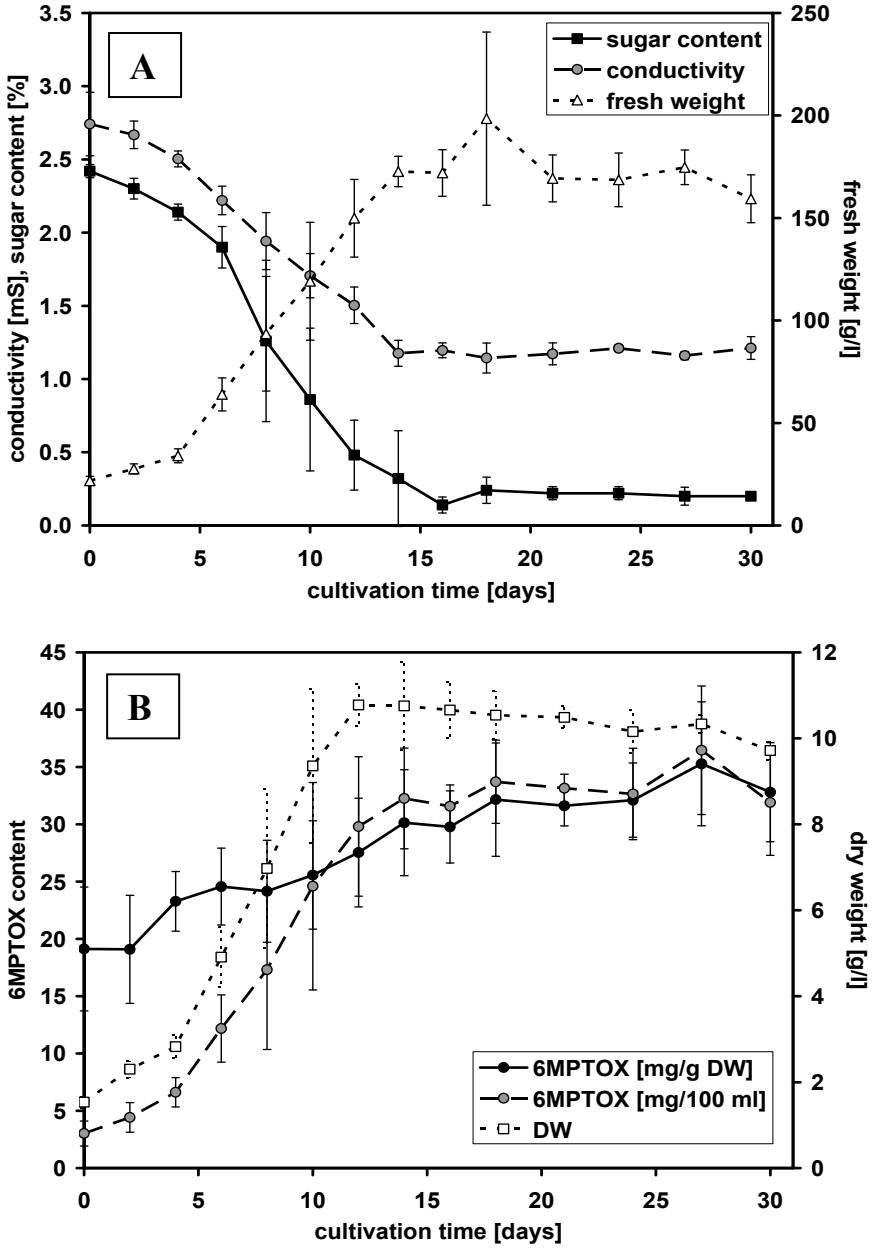


Fig. 19: Growth and lignan accumulation of a hairy root line from *Linum album* (line 3-4) during a cultivation period of 30 days (data are mean +/- SD of three independent measurements). The parameters measured were:
A: Growth of the culture measured as fresh weight, sugar content and conductivity of the medium, B: Growth of the culture measured as dry weight, 6MPTOX content of the cells

Since these first experiments we screened 15 different shoot cultures of *L. album* for their sensitivity against *A. rhizogenes* to optimize the transformation protocol because the main limitations in the old protocol were the availability of seeds and their germination rate. According to the fact that only few *L. album* seedlings were susceptible for transformation from these 15 shoot culture only two showed transformation rates of about 7-20 % in different experiments. In following experiments these shoot cultures will be used.

In our next attempts we were able to establish hairy roots from other *Linum* species which accumulate other lignans than *L. album* or *L. persicum* by using the protocol established with *L. album*. Most *Linum* species showed highest sensitivity for *A. rhizogenes* strain TR105. The hairy roots usually accumulate the same lignans in either comparable or higher amounts than cell cultures (Tab. 1).

Tab. 1: Lignan accumulation in hairy roots in comparison to cell cultures

plant species	lignan	lignan content [mg/g dry weight] in hairy roots (ref.)	lignan content [mg/g dry weight] in cell cultures (ref.)
L. flavum	6MPTOX	20.0 ± 10.1 at day 22 * (Oostdam et al. 1993)	3.0 at day 14 (Oostdam et al. 1993)
L. album	6MPTOX	40.1 ± 2.5 at day 22 * (will be published soon)	4.0 ± 1.7 at day 14 * (will be published soon)
L. persicum	6MPTOX	49.1 ± 5.5 at day 26 * (will be published soon)	not determined
L. corymbulosum	HINO	0.28 ± 0.0 at day 12 * (Mohagheghzadeh et al. 2006)	0.36 ± 0.1 at day 14 § (Mohagheghzadeh et al. 2006)
L. austriacum	JusB	16.9 at day 30 (Mohagheghzadeh et al. 2002)	6.7 at day 14 Mohagheghzadeh et al. 2002)
L. perenne var. Himmelszelt	JusB	36 ± 2.3 at day 14 § (Hemmati et al. in press)	19.8 ± 3.1 at day 3.1 * (Hemmati et al. in press)

*The values are means +/- SD from several independent lines.

§The values are means +/- SD from several measurements on one line.

In addition to the hairy roots mentioned in Tab. 1 we recently could establish hairy root cultures from *L. usitatissimum*, *L. bienne* and *L. grandiflorum*. But, we could not detect lignan accumulation in theses lines up to now although plants of *L. usitatissimum* and *L. bienne* were shown to accumulate lignans (Schmidt et al. 2006).

It is remarkable that hairy roots from *Linum* species with stable plants (e. g. *L. usitatissimum*, *L. album*, *L. grandiflorum*) usually can be established easier and grow faster than hairy root cultures from *Linum* species which grow as relatively small plants with thin shoots (e. g. *L. bienne*, *L. corymbulosum*). Auxin in a concentration of 1 mg/l can be added to the cultivation medium in the first passages after removing the hairy roots from the explant to overcome these problems in the first passages according to Park and Facchini (2000).

4.3. Genetic manipulation of the lignan biosynthesis in hairy roots

The gene transfer mechanisms of *A. rhizogenes* is very similar to the one of *A. tumefaciens* which is very well understood and elaborated for foreign gene transfer to plants. Therefore, foreign genes can be introduced in hairy roots by using the highly elaborated vectors for *A. tumefaciens*.

After establishment of the transformation protocol for *L. album* with *A. rhizogenes* we were interested to test whether foreign gene constructs based on vectors for *A. tumefaciens* can be cotransformed with the Ri-plasmid.

The antibiotics kanamycin, paromomycin and hygromycin and the herbizide BASTA® were added into the solid medium of hairy roots without foreign gene constructs and growth followed over 4 weeks to test whether they are suitable as selection agent. Kanamycin had no influence on the growth even in concentrations as high as 500 mg/l. The more effective paromomycin could inhibit the growth in a concentration of 200 mg/l. 100 mg/l hygromycin or BASTA® was effective as well.

The pinoresinol-lariciresinol reductase (PLR) is supposed to be involved in the biosynthesis of PTOX, 6MPTOX and α-peltatin (Fig. 4). We cloned a cDNA encoding a PLR from *L. album*. The open reading frame of this gene was used to build an intron containing "hairpin" RNA (ihpRNA) construct according to Wesley et al. (2001) (Fig. 20). The expression of such constructs leads to the formation of double stranded RNA which reduces mRNA levels of the target gene via the RNAi mechanism. Explants of one susceptible shoot culture of *L. album* were either cotransformed with a binary vector (pART27) containing this construct or an empty vector control or only transformed with *A. rhizogenes* strain 15834 (ATCC).

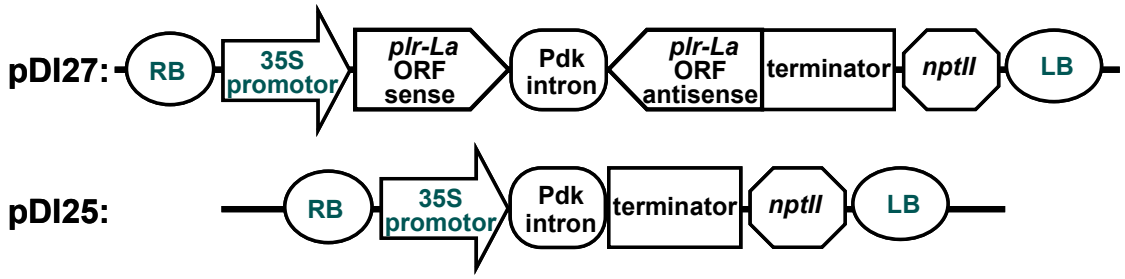


Fig. 20: Constructs for the transformation of *L. album*

Six independent hairy root lines carrying the ihpRNA construct (PDI27), four controls with the empty vector (PDI25) and five controls without construct (ATCC) showed comparable growth and typical hairy root morphology. They were further analysed after removing the agrobacteria by addition of 100 mg/l timentin® to the medium shown by PCR for the *virC* gene. The hairy root status was proven by amplification of a fragment of the *rolC* gene.

PDI25 and ATCC lines accumulate app. 30 mg/g dry weight 6MPTOX and α-peltatin (not quantified) indicating no influence of the empty vector on lignan biosynthesis (Fig. 21). In contrast, no α-peltatin and only app. 5 mg/g dry weight 6MPTOX were accumulated in PDI27

lines. Only in these lines pinoresinol and lariciresinol were detectable. This indicates the silencing effect on *plr* gene expression by the *plr-La* ihpRNA construct.

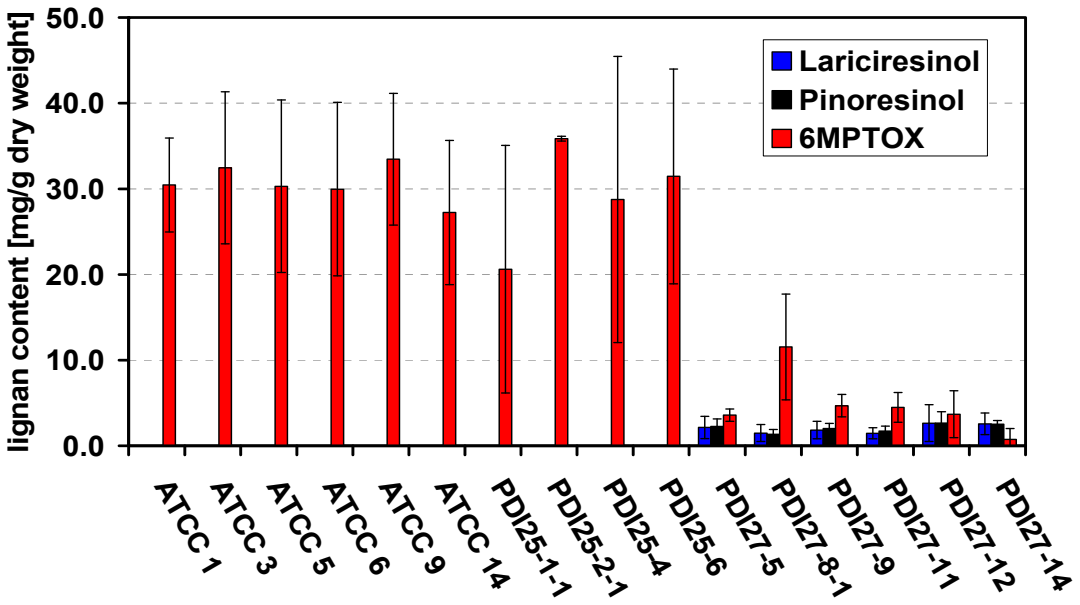


Fig. 21: Lignan content of hairy roots lines without (ATCC), with empty vector (PDI25) and *plr-La1* ihpRNAi construct (PDI27) after a cultivation period of 14 days (The values are mean +/- SD from three determinations within 5 month.)

Transcript levels of *plr-La1* were measured in silenced lines in comparison to control lines. In all ihpRNA lines the level of *plr-La1* mRNA was significantly reduced (Fig. 22). The levels of the specific activity of PLR were reduced from 40 nkat/kg protein in control lines to 10 nkat/kg protein in ihpRNA lines (Fig. 22). This is the first proof on the molecular level that PLR is involved in the biosynthesis of PTOX and α-peltatin.

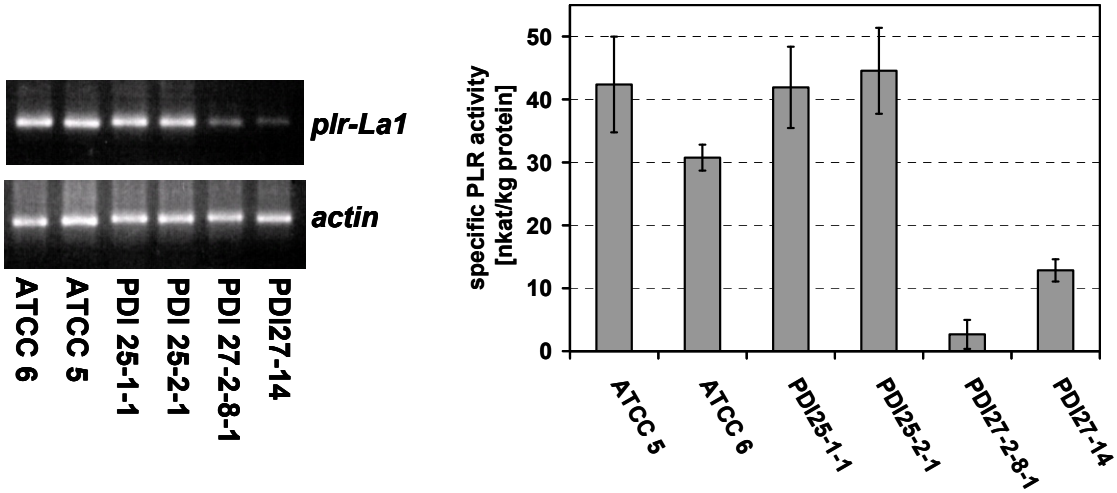


Fig. 22: left: RT-PCR analysis of *plr-La1* gene expression in *L. album* hairy roots. An *actin* gene from the same lines was amplified to determine equal amounts of cDNA template
right: PLR activity with 40 µg protein and 30 min incubation in the same lines as chosen for the RT-PCR analysis containing no foreign vector (ATCC), an empty vector (PDI25) and the *plr-La1* ihpRNAi construct (PDI27) (The values are mean +/- SD from two times assays conducted in triplicate.)

An analogous experiment was conducted with *L. perenne* var. Himmelszelt shoot cultures and an ihpRNA construct to silence the *plr-Lp1* gene (Hemmati et al. in press). Six independent lines carrying the ihpRNA construct (H), five an empty vector (E) and six without construct (T) were obtained. All lines showed comparable growth and hairy root morphology. The mRNA levels for the *plr-Lp1* gene as well as the PLR activity was significantly reduced in the lines carrying the ihpRNA construct (Fig. 23).

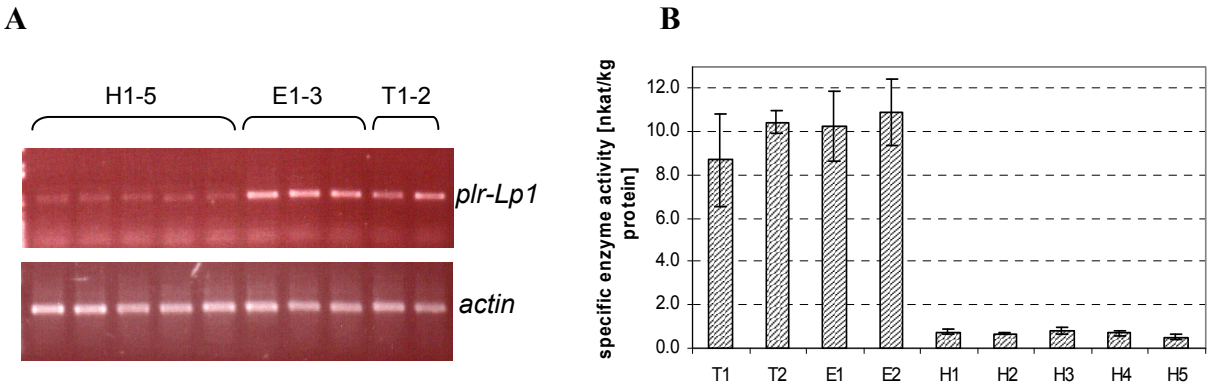


Fig. 23. **A**: RT-PCR analysis of *plr-Lp1* gene expression in *L. perenne* hairy roots. An *actin* gene from the same lines was amplified to determine equal amounts of cDNA template.
B: PLR activity with 40 µg protein and 90 min incubation in selected hairy root lines of *L. perenne* containing *plr* ihpRNAi constructs (H) in comparison with lines containing empty vector (E) and lines without any vector (T). (The values are mean±SD from two times assays conducted in duplicate.)

L. perenne var. Himmelszelt control lines E and T accumulate 29-38 mg JusB as major lignan accompanied by two diphyllin glycosides indicating no influence of the control construct on the lignan biosynthesis (Fig. 24). The level of the diphyllin glycosides and JusB was significantly reduced ton 6-11 mg/g dry weight JusB in the ihpRNA lines.

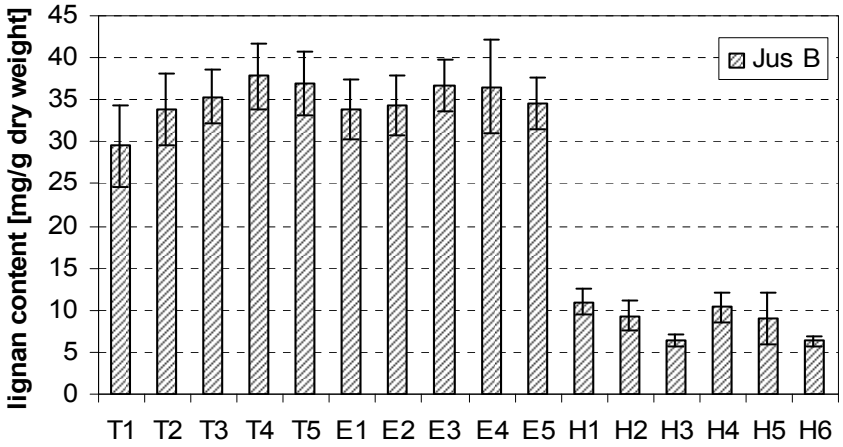


Fig. 24. Justicidin B (JusB) content in hairy roots of *L. perenne* Himmelszelt: (T1-5): lines without any construct, (E1-5): lines with empty vector, (H1-6): lines with ihpRNAi construct. The lignan content was determined in triplicate.

In contrast to *L. album plr*-ihpRNA lines *L. perenne plr*-ihpRNAi lines accumulate only minor amounts of pinoresinol. Instead, two new peaks were observed in the ihpRNA lines by HPLC analysis. LC-MS data suggest that the substances could be glycosides of pinoresinol and lariciresinol. It is possible that Pinoresinol and Lariciresinol can serve as substrates for the same enzymes which add the sugar moieties to the diphyllins accumulated in the *in vitro* cultures of *L. perenne* Himmelszelt. Nevertheless, the exact structures of the compounds still remain to be identified. In conclusion our results demonstrate that a PLR is involved in the biosynthesis of arylnaphthalene type lignans like JusB.

4.4. Conclusion

The fast growing hairy root cultures from *Linum album* (5 days doubling time) and *L. persicum* show the highest accumulation of the cytotoxic 6MPTOX (up to 5.7 % of the DW) which has ever been observed in *in vitro* cultures. The variability of the 6MPTOX content within the different lines generated from also different seedlings is not very high. This is a good starting point to conclude the influence of up- or downregulation of possible genes maybe involved in 6MPTOX biosynthesis. A disadvantage would be the availability of the seed material and the sometimes very low germination rates. Therefore, we established shoot cultures as starting material for the transformation experiments. For this purpose 15 lines were screened to obtain appropriate plant material since not more than 40 % of the infected seedlings gave hairy roots. Two shoot cultures were susceptible. One was used for a first experiment to genetically manipulate the lignan biosynthesis in hairy roots of *L. album*. The introduced ihpRNA construct with the open reading frame of the *plr-La* gene led to a significant decrease of PLR activity followed by lowered levels of the arylteraline type lignans 6MPTOX and α-peltatin in comparison to control lines. This is the first proof by molecular biological methods that the PLR is involved in the biosynthesis of these lignans.
The transformation protocol can be easily adapted to other *Linum species*. Similar experiments with an ihpRNA construct containing the open reading from of the *plr-Lp* gene in *L. perenne* var. Himmelszelt hairy roots proofed that the PLR is also involved in the biosynthesis of arylnaphthalene type lignans like JusB and diphyllins.
These experiments are the first experiments to genetically manipulate the lignan biosynthesis. The established protocols for gene silencing can be used to test newly cloned genes for their contribution in lignan biosynthetic pathways. Furthermore, since the ihpRNAi constructs cause a block in the early steps of lignan biosynthesis the obtained lines can be used to feed possible intermediates in lignan biosynthesis after action of PLR in order to give strong hints for their involvement in the particular pathway. E. g. deoxypodophyllotoxin is converted to 6MPTOX by the ihpRNA lines of *L. album*.

5. Stereospecificity in lignan biosynthesis: Pinoresinol-lariciresinol reductase

von Heimendahl CBI, Schäfer K, Eklund P, Sjöholm R, Schmidt TJ, Fuss E* (2005): Pinoresinol-lariciresinol reductases with different stereospecificity from *Linum album* and *Linum usitatissimum*. Phytochemistry 66, 1254-1263

Hemmati S, Schmidt TJ, Fuss E*: (+)-Pinoresinol/(-)-lariciresinol reductase from *Linum perenne* Himmelszelt involved in the biosynthesis of justicidin B (FEBS Letters, in press)

Hemmati S, von Heimendahl CBI, Fuss E*: Pinoresinol-lariciresinol reductases with opposite enantiospecificty determine the enantiomeric composition of lignans in *Linum usitatissimum* (will be submitted soon)

Von Heimendahl CBI, Fuss E*: The molecular basis of different enantiospecificity of pinoresinol-lariciresinol reductases (in preparation)

Von Heimendahl CBI, Treml M, Fuss E*: Pinoresinol-lariciresinol reductases and related proteins from *Arabidopsis thaliana* (in preparation)

* corresponding author

5.1. Introduction

Most lignans are chiral compounds. In each plant or organ only one enantiomer can be found. The enantiomeric purity seems to be determined at different levels in lignan biosynthesis. Already the coupling of the two achiral coniferyl alcohol molecules with aid of the dirigent protein leads in *Forsythia intermedia* to enantiomeric pure (+)-pinoresinol [(+)-PINO] which has R,R-configuration at C-atoms 8,8' (Davin and Lewis 2003). In contrast, enantiomeric purity is reached on the level of matairesinol (MATAI) in *Wikstroemia sikokiana* (Umezawa 2003). Interestingly, opposite lignan enantiomers can be found in different plants or organs. Enzyme preparations of flowers of *Arctium lappa* catalyse the formation of (+)-PINO, (+)-lariciresinol [(+)-LARI] and (-)-SECO whereas enzyme reparations from ripening seeds of this plant species catalyse the formation of the opposite enantiomers (Suzuki et al. 2002). Seeds of *L. usitatissimum* contain pure (+)-secoisolariciresinoldiglucoside whereas *L. album* accumulates pure (-)-PTOX which should have (-)-secoisolariciresinol [(-)-SECO] as precursor (Fig. 25) (Davin and Lewis 2003; Petersen and Alfermann 2001).

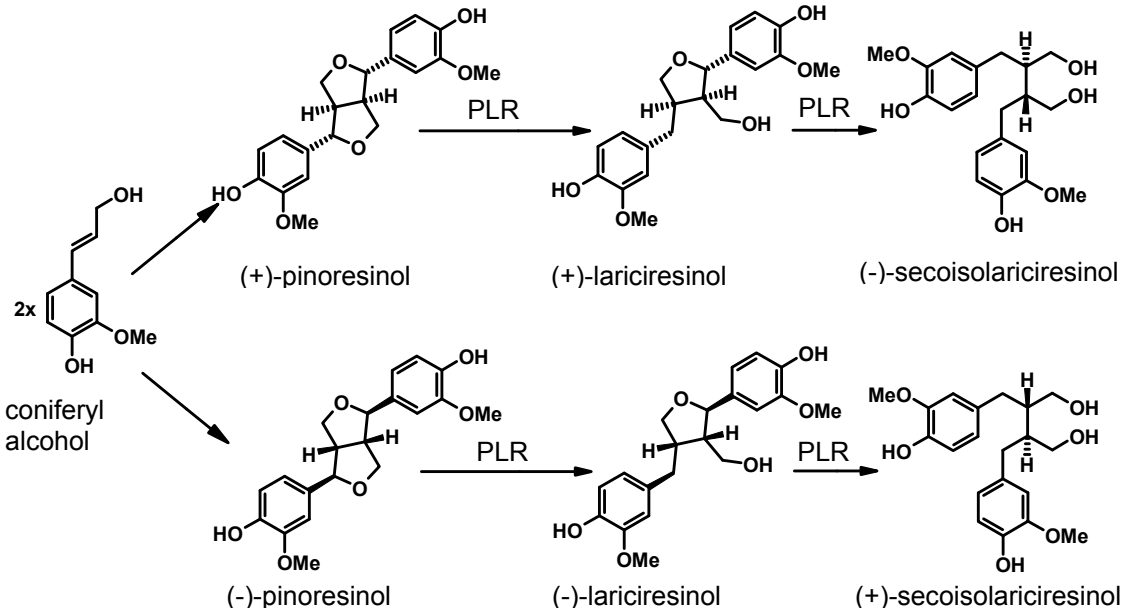


Fig. 25: The first steps in lignan biosynthesis with opposite enantiospecificity (PLR = pinoresinol-lariciresinol reductase)

Since *Linum* species are a rich source for lignans in general and since *L. album* and *L. usitatissimum* is a good example of accumulation of lignans with opposite enantiomeric composition. We decided to start a project on the development on lignan biosynthesis in Linum species with one focus on the stereospecificity.

According to the recently established molecular phylogeny of the Linaceae (Kadereit, Repplinger, unpublished results, Fig. 26) the genus *Linum* can be divided in two main clusters, one containing the section Syllinum, the other the section Linum. In order to compare the molecular phylogeny with the lignan content in *Linum* species we investigate cultures and plants from several *Linum* species of the different phylogenetic groups in cooperation with B. Konuklugil (Turkey), I. Ionkova (Bulgaria), T. J. Schmidt (Münster) and A.W. Alfermann (Konuklugil et al. 2005; Konuklugil et al. 2007; Ionkova et al. 2007; Vasilev et al. submitted, Ionkova et al.; submitted). Aryltetralin lignans were found in cultures and plants of section Syllinum whereas no, simple lignans or arylnaphthalene lignans were found in section Linum (Fig. 26). There is only one exception of this general rule up to now. Small amounts of the arylnaphthalene lignan justicidin B beside the arylteralines PTOX and 6MPTOX were detected in *L. tauricum* ssp. *linearifolium* from section Syllinum indicating that the capability for the formation of aryltertralins as well as arylnaphthalenes was already present in the evolutionary ancestor of all *Linum* species. Specialisation for the formation of either aryltetralin or arylnaphthalene lignans occurred in evolution during separation of the two main groups of *Linum* species containing the section Syllinum or Linum (Schmidt TJ, Hemmati S, Fuss E, Konuklugil B, Mohagheghzadeh A, Ionkova I, Alfermann AW: Chemodiversity of lignans in the genus *Linum*, in preparation).

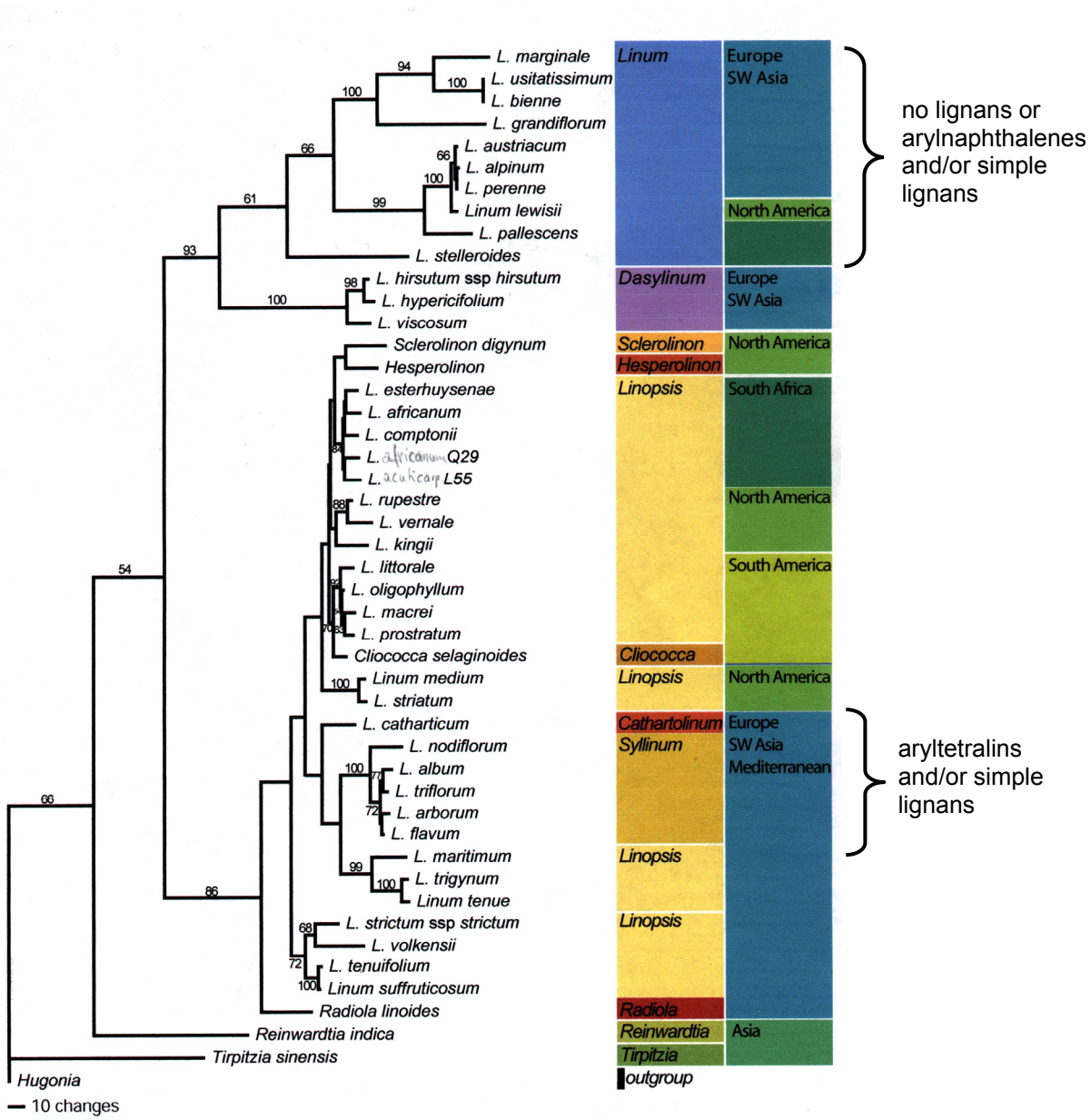


Fig. 26: Phylogenetic tree of the genus *Linum* based on ITS-analytics, figure with permission of Prof. Dr. J. Kadereit & M. Repplinger, Institut für Spezielle Botanik, Universität Mainz, together with occurance of lignans

5.2. Enantiospecificity in lignan biosynthesis of Linum species

As mentioned above different *Linum* species accumulate lignans of different enantiomeric composition. In a first attempt we would like to figure out, at which level enantiomeric purity is reached in lignan biosynthesis in *Linum* species. We collected the lignans from cell cultures of *L. album* and *L. usitatissimum* and subjected them to chiral column analysis (Fig. 27) (von Heimendahl et al. 2005).

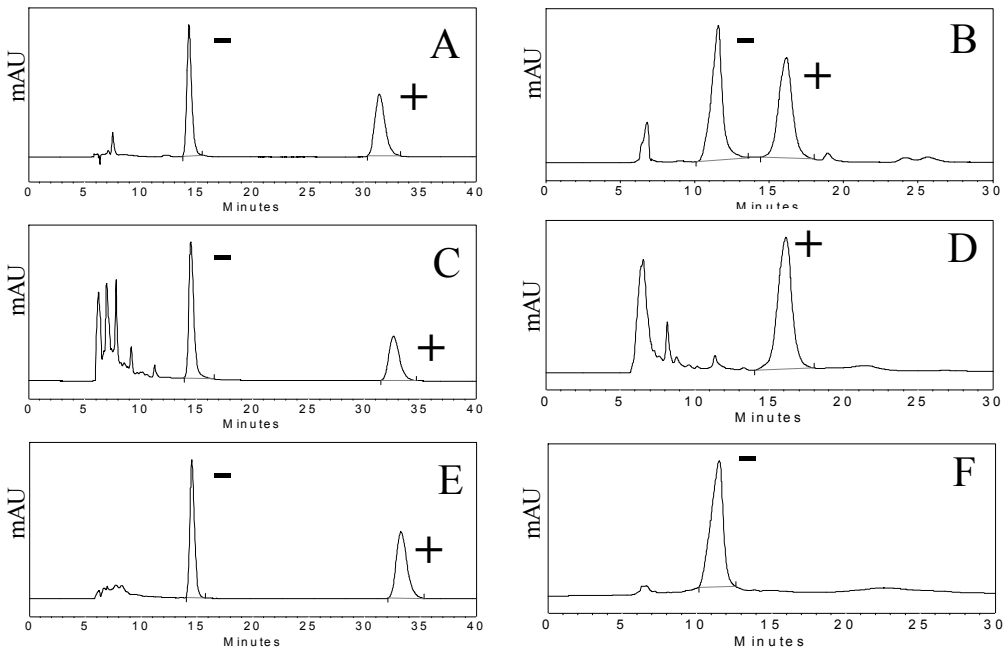


Fig. 27: Enantiomeric composition of pinoresinol (C, E) and secoisolariciresinol (D, F) in cell cultures from *L. album* (E, F) and *L. usitatissimum* (C, D) in comparison to racemic standards (A: pinoresinol, B: secoisolariciresinol) at day 12 and 10 respectively (von Heimendahl et al. 2005)

Whereas PINO was found in both cultures as mixture of both enantiomers, pure (-)-SECO was found in *L. album* and pure (+)-SECO in *L. usitatissimum* cell cultures. We conclude that enantiomeric purity of lignans is determined by the action of the pinoresinol-lariciresinol reductase (PLR) in these *Linum* species. In the hairy root lines which are carrying the ihpRNA construct to downregulate the PLR activity PINO accumulates as enantiomeric mixture and LARI as pure (+)-enantiomer indicating that already the first step of the PLR reaction is enantioselective (S. Steegmüller).

We cloned a full length cDNA encoding a PLR from *L. album* and an open reading frame of a PLR from *L. usitatissimum* (von Heimendahl et al. 2005). The proteins were expressed as 6His fusion proteins in *E. coli*, purified and their enantiospecificity was determined. For that purpose assays with different protein concentrations were conducted with racemic PINO as substrate (C.B.I. von Heimendahl). The remaining PINO and the newly formed LARI and SECO were collected during HPLC analysis on reversed phase and investigated for their enantiomeric composition by using chiral phase HPLC (Fig. 28). Analogous experiments were conducted with variation of the reaction time resulting in comparable courses of PINO consumption and formation of LARI and SECO.

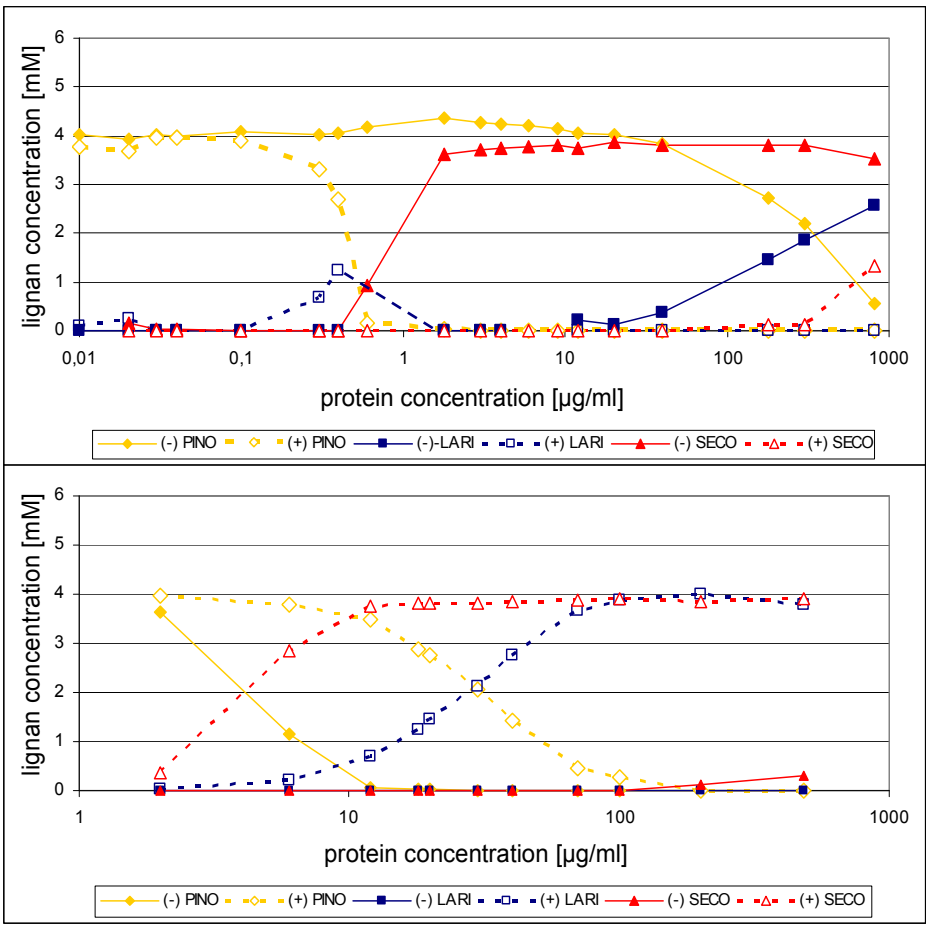


Fig. 28: Enantiomeric composition of lignans in assays with racemic PINO as substrate with purified PLR from *L. album* (upper panel) and *L. usitatissimum* (lower panel) (One example of three independent determinations is shown)

At first the PLR from *L. album* uses (+)-PINO to form (+)-LARI followed by the formation of (-)-SECO. The same result was observed by following the enantiospecificty of PLR activity in raw extracts of hairy roots (Fig. 29) (S. Steegmüller). The PLR strongly prefers (+)-PINO to form (-)-SECO via (+)-LARI. The heterologously expressed and purified PLR converts also (-)-PINO to (-)-LARI and (-)-SECO at high protein concentrations and after long reaction times. The accumulation of LARI as intermediate indicates that between both reactions catalysed by the PLR the reaction from LARI to SECO is rate limiting in the formation of SECO. The PLR from *L. usitatissimum* first of all uses (-)-PINO to form (+)-SECO immediately indicating that the reaction from (-)-PINO to (-)-LARI is rate limiting for the formation of (+)-SECO. The reactions from (+)-PINO to (+)-LARI to (-)-SECO can be observed with high protein concentrations after long incubation times. Therefore, we cloned the first PLRs which are able to form both enantiomers of SECO. The PLRs cloned so far from *Forsythia intermedia* and *Thuja plicata* are highly enantiospecific for the formation of either (-)-SECO or (+)-SECO although PLRs from *Thuja plicata* are not highly enantioselective for the PINO enantiomers (Dinkova-Kostova et al. 1996; Fujita et al. 1999).

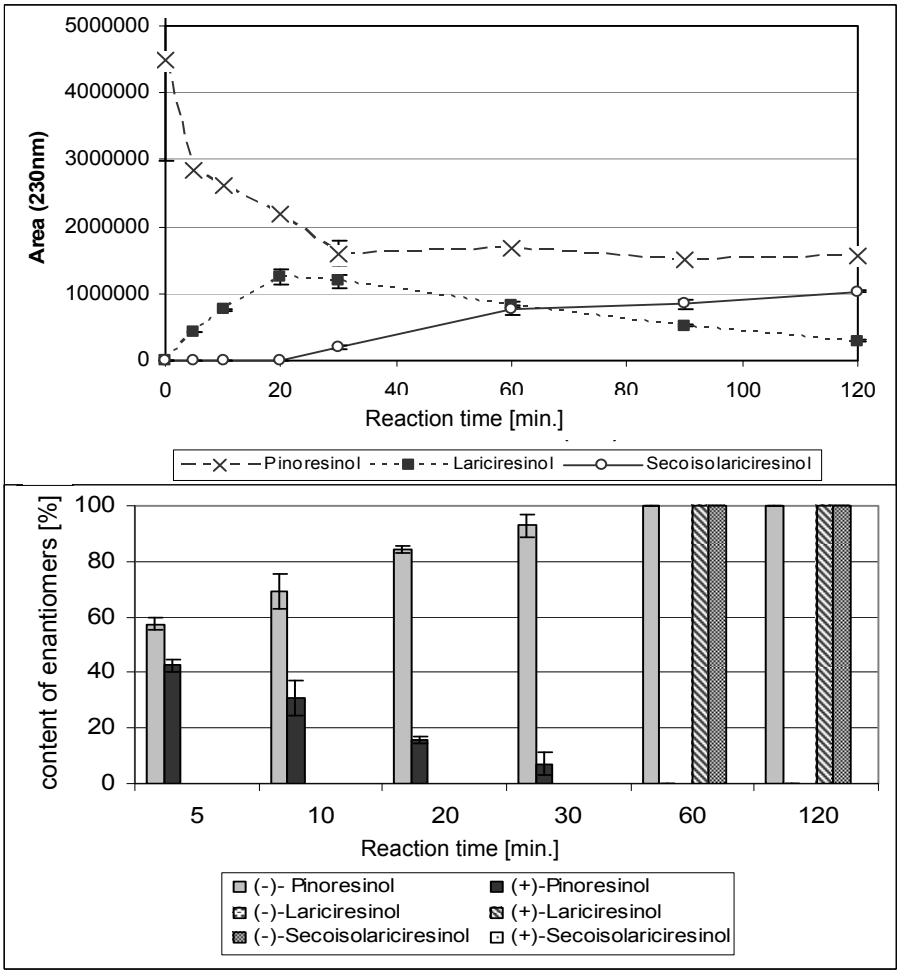


Fig. 29: upper part: consumption of PINO and formation of LARI and SECO over the reaction time in PLR assays with 40 µg/500 µl assay protein from hairy roots, line 3-4 and racemic PINO as substrate (The data are the mean of two independent experiments.)
lower part: enantiomeric composition of PINO, LARI and SECO in the enzyme assays

Southern blot analysis (Fig. 30) indicated that the genome of *L. album* contains one *plr* gene copy which fits to the enantiomeric purity of the lignans starting from LARI in the biosynthetic pathway to PTOX and 6MPTOX which are pure (-)-enantiomers (Petersen and Alfermann 2001; E. Fuss, T. Gulder (Universität Würzburg) and S. Steegmüller, unpublished results). The strong enantiospecificity of the cloned PLR for the formation of (-)-SECO fits to these results.

In contrast, a small *plr* gene family is present in the genome of *L. usitatissimum*. Because the SECOdiglucoside in the seeds of *L. usitatissimum* is only 99 % pure (+)-enantiomer, one could assume that at least a second PLR with opposite enantiospecificity to the one we have cloned is encoded in the genome. Recently, lignans up to yatein with the same enantiomeric composition like the lignans in *L. album* were isolated from flowering plants of *L. usitatissimum* giving further hints for the demand of a second *plr* gene (Schmidt et al. 2006).

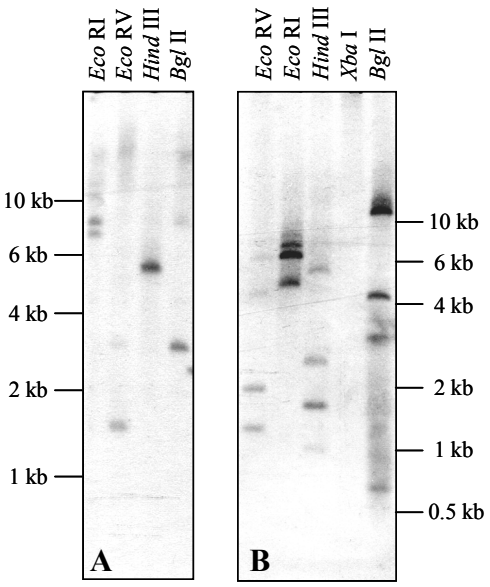


Fig. 30: Southern hybridisation of *L. album* and *L. usitatissimum* genomic DNA. DNA was digested with *Eco* RI, *Eco* RV, *Hind* III, *Bgl* II and only gDNA of *L. usitatissimum* with *Xba* I. The resulting membranes were probed with the open reading frames of *PLR-La1* (A) and *PLR-Lu1* (B), respectively

Indeed, recently we cloned a second cDNA encoding a PLR from leaves of flowering *L. usitatissimum* plants (Hemmati and Fuss, publication in preparation). The protein was expressed in *E. coli* as 6His fusion protein and investigated for its enantiospecificity. In contrast to the first PLR, the second PLR from *L. usitatissimum* strongly prefers (+)-PINO to form (-)-SECO and therefore has opposite enantiospecificity to the PLR cloned before. Semi quantitative PCR analysis revealed that the first PLR is only expressed in seeds whereas the second PLR is expressed in seeds as well as leaves from flowering plants (Fig. 31). This indicates that the enantiomeric composition of lignans is determined by the expression levels of the *plr* genes in *L. usitatissimum*.

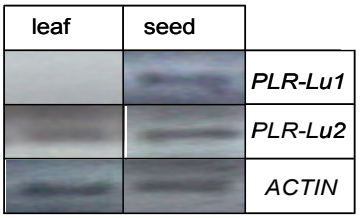


Fig. 31: semi quantitative RT-PCR analysis of gene expression of *PLR-Lu1*, specific for (-)-PINO, and *PLR-Lu2*, specific for (+)-PINO, in leaves of flowering plants and developing seeds. An *actin* gene was used as internal control. 10 µl of 29-cycle PCR products were loaded on 1 % (w/v) agarose gel

5.3. Molecular basis for different enantiospecificities of PLRs

The amino acid sequences of PLRs with opposite enantiospecificities were compared in order to identify amino acid positions which could be responsible for the different enantiospecificity of PLRs. In four positions of PLRs which produce only (-)-SECO (e.g. PLR-

Tp2, PLR-La1) one amino acid is highly conserved whereas in PLRs which produce only (+)-SECO (e.g. PLR-Tp1, PLR-Lu1) another amino acid is conserved. These amino acids are: Met[143], Gly[282], Tyr[286] and Val[318] in PLR-LA instead of Leu[129], Val[268], Leu[272] and Met[304] in PLR-Lu1. Recently the crystal structure of a PLR from *Thuja plicata* was elucidated (Min et al., 2003). In comparison to this structure Val[268] and Leu[272] (PLr-Lu1) are in the binding pocket for the substrate and also suggested by Min et al. as involved in enantiospecificity. Leu[129] and Met[304] are at the borders of the pocket. Additionally, Min et al. suggested the position Phe[164] (PLR-Tp2) as possibly involved in enantiospecificity, but in our sequence comparisons also PLRs with the opposite specificity have a Phe at the comparable position.

```
PLR-La1                MGSLGKVNNEIPTKSSGGSKVLVIGGTGYLGKRLVKASLDSGHDTYVMHRPE 52
PLR-Fi1                --------------MGKSKVLIIGGTGYLGRRLVKASLAQGHETYILHRPE 37
PLR-Tp2                --------------MEESSRVLIVGGTGYIGRRIVKASIALGHPTFILFRKE 38
PLR-Lu1                ---------------MGRCRVLVVGGTGYIGKRIVKASIEHGHDTYVLKRPE 37
PLR-Tp1                --------------MDKKSRVLIVGGTGYIGKRIVNASISLGHPTYVLFRPE 38
                                     ========

PLR-La1          ---IGVDIEKVQLLLSFKMQGAHLVSASFDDQRSLVDAVKLVDVVICAISGVHIRSHQIL 109
PLR-Fi1          ---IGVDIDKVEMLISFKMQGAHLVSGSFKDFNSLVEAVKLVDVVISAISGVHIRSHQIL 94
PLR-Tp2          ---VVSDVEKVEMLLSFKKNGAKLLEASFDDHESLVDAVKQVDVVISAVAGNHMRHH-IL 94
PLR-Lu1          ---TGLDIEKFQLLLSFKKQGAHLVEASFSDHESLVRAVKLVDVVICTVSGAHSRS--LL 92
PLR-Tp1          ---VVSNIDKVQMLLYFKQLGAKLIEASLDDHQRLVDALKQVDVVISALAGGVLSHH-IL 94

                                                 •
                                                 ■
PLR-La1          LQLKLVEAIKEAGNVKRFVPSEFGTDPAR-MENAMEPGRITFDDKMVVRRAIEEAGIPFT 168
PLR-Fi1          LQLKLVEAIKEAGNVKRFLPSEFGMDPAKFMDTAMEPGKVTLDEKMVVRKAIEKAGIPFT 154
PLR-Tp2          QQLKLVEAIKEAGNIKRFVPSEFGMDPGL-MEHAMAPGNIVFIDKIKVREAIEAASIPHT 153
PLR-Lu1          LQLKLVEAIKEAGNVKRFIPSEFGMDPAR-MGDALEPGRETFDLKMVVRKAIEDANIPHT 151
PLR-Tp1          EQLKLVEAIKEAGNIKRFLPSEFGMDPDI-MEHALQPGSITFIDKRKVRRAIEAASIPYT 153

                       ▼
PLR-La1          YVSANCFAGYFLGGLCQPGY---ILPSRDHVTLLGDGDKKGVYVDEDDTAAYTLRAIDDP 225
PLR-Fi1          YVSANCFAGYFLGGLCQFGK---ILPSRDFVIIHGDGNKKAIYNNEDDIATYAIKTINDP 211
PLR-Tp2          YISANIFAGYLVGGLAQLGR---VMPPSEKVILYGDGNVKAVWVDEDDVGIYTIKAIDDP 210
PLR-Lu1          YISANCFGGYFVGNLSQLGP---LTPPSDKVTIYGDGNVKVVYMDEDDVATYTIMTIEDD 208
PLR-Tp1          YVSSNMFAGYFAGSLAQLDG--HMMPPRDKVLIYGDGNVKGIWVDEDDVGTYTIKSIDDP 211

                                                          ▼
                                                          ■
PLR-La1          RTLNKTIYVKPPKNVLSQREVVGIWEKYIGKELQKTILSEQDFLATMREQNYAEQVGLTH 285
PLR-Fi1          RTLNKTIYISPPKNILSQREVVQTWEKLIGKELQKITLSKEDFLASVKELEYAQQVGLSH 271
PLR-Tp2          HTLNKTMYIRPPLNILSQKEVVEKWEKLSGKSLNKINISVEDFLAGMEGQSYGEQIGISH 270
PLR-Lu1          RTLNKTMYLRPPENVITHRQLVETWEKLSGNQLQKTELSSQDFLALMEGKDVAEQVVIGH 268
PLR-Tp1          QTLNKTMYIRPPMNILSQKEVIQIWERLSEQNLDKIYISSQDFLADMKDKSYEEKIVRCH 271

                 ▼                                 •
                 ■                                 ■
PLR-La1          YYHVCYEGCLSNFEVD---DEQEASKLYPDVHYTTVEEYLKRYV 326
PLR-Fi1          YHDVNYQGCLTSFEIG---DEEEASKLYPEVKYTSVEEYLKRYV 312
PLR-Tp2          FYQMFYRGDLYNFEIGP--NGVEASQLYPEVKYTTVDSYMERYL 312
PLR-Lu1          LYHIYYEGCLTNFDIDAAQDQVEASSLYPEVEYIRMKDYLMIYL 312
PLR-Tp1          LYQIFFRGDLYNFEIGP--NAIEATKLYPEVKYVTMDSYLERYV 313
```

Fig. 32: Amino acid sequence alignment between PLRs with specificity to form (-)-SECO (blue) from *Linum album* (PLR-La1, accession no. AJ849358), *Forsythia intermedia* (PLR-Fi1, accession no. U81158) and *Thuja plicata* (PLR-Tp2, accession no. AF242504) and PLRs with specificity to form (+)-SECO (red) from *Linum usitatissimum* (PLR-Lu1, accession no. AJ849359) and *Thuja plicata* (PLR-Tp1, accession no. AF242503) The conserved sequence “GxxGxxG” of the NADPH binding domain is double underlined. Black squares and bold letters indicate amino acid positions with differently conserved amino acids in PLRs with different stereochemistry. Black dots indicate amino acid positions which are located only near to the catalytic centre of PLRs according to Min et al. (2003). Black triangles indicate amino acid positions which are discussed to be involved in stereospecificity by Min et al. (2003).

The PLR of *L. album* was altered by site-directed mutagenesis to change its enantiospecificity. Primers were designed such that Gly^{282} was changed to Val, Tyr^{286} to Leu and Val^{318} to Met. The heterologously expressed protein prefers (-)-PINO and converts it to (-)-LARI and then (+)-SECO, but still can use (+)-PINO and forms (+)-LARI too (Fig. 33). The enantiospecificity was almost completely changed in the mutant. Therefore, the amino acids Gly^{282}, Tyr^{286} and Val^{318} are responsible but not only sufficient to determine enantiospecificity of PLRs (C.B.I. von Heimendahl, E. Fuss, publication in preparation).

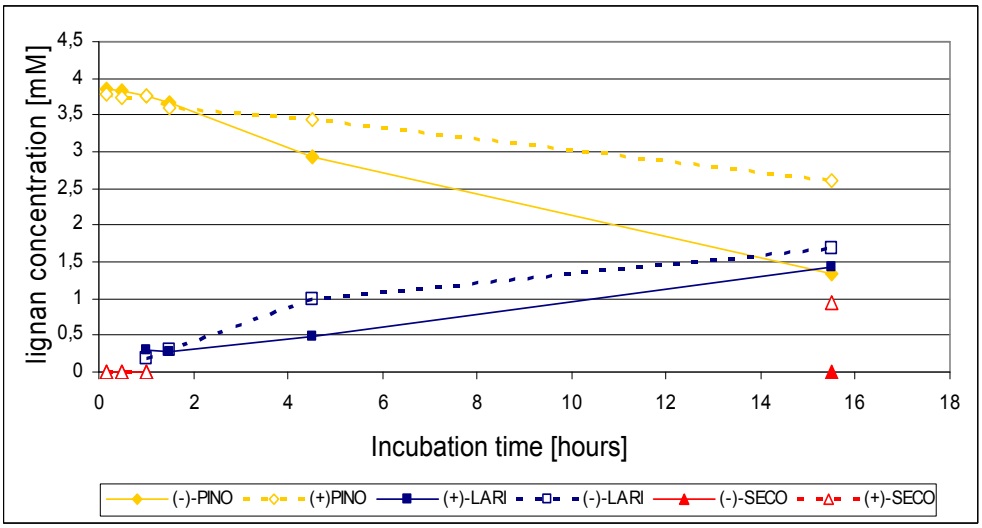


Fig. 33: Enantiomeric composition of lignans in assays with racemic PINO as substrate with purified mutant PLR from *L. album*

5.4. Evolution of PLRs and their enantiospecificity

PLRs belong to the so called PIP-family of reductases (Koeduka et al. 2006). The members of this family who are responsible for its name are: **P**LRs, **i**soflavone reductases (IFRs) and **p**henylcumaran benzylic ether reductases (PCBERs). PLRs, PCBERs and IFRs do not only show high sequence similarities but also comparable reaction mechanisms (Fig. 34) (Gang et al. 1999). Whereas PLRs and IFRs are stereospecific, PCBERs are only regioselective. Gang et al. (1999) supposed that PCBERs are the progenitors for the widely distributed PLRs and IFRs which are almost only found in the Fabaceae.

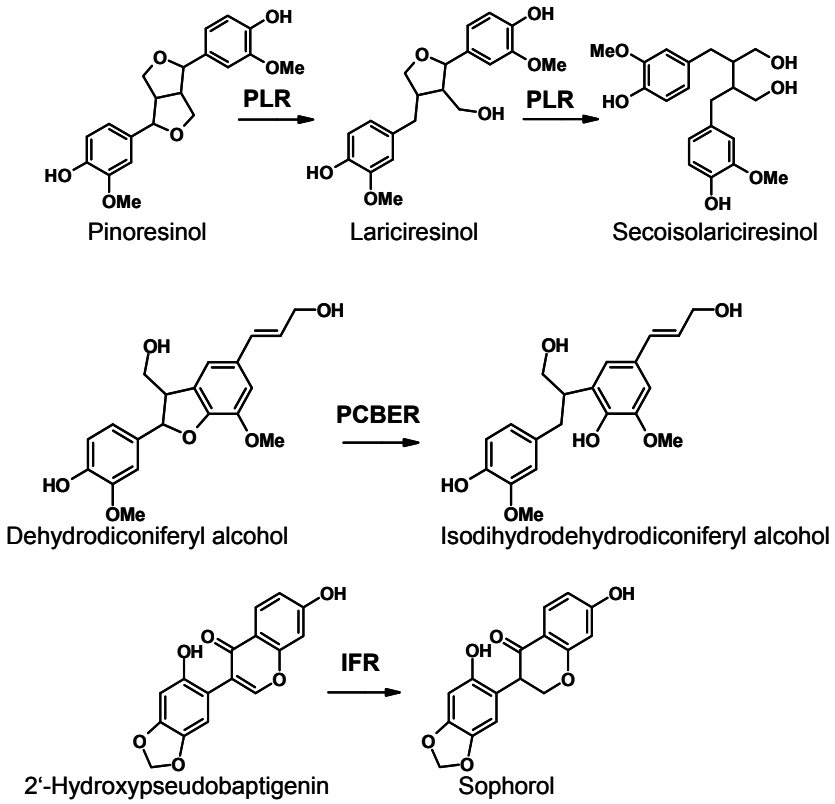


Fig. 34: Reactions catalysed by PLRs, PCBERs and IFRs

For a better understanding of the PLRs and their evolution we cloned several cDNAs of *Linum* species encoding PLRs and PCBERs (see chapters before) as well as members of the PIP family in *Arabidopsis thaliana*. We have identified 8 members of the PIP family in *A. thaliana* (C.B.I von Heimendahl, M. Treml, S. Wagner, E. Fuss, publication in preparation). Almost all were heterologously expressed in *E. coli*, purified and their function determined in assays with PINO as substrate for PLRs and dehydrodiconiferyl alcohol as substrate for PCBERs. AT1 and AT2 are PLRs, but AT1 has some PCBER activity too and is therefore the first PLR with PCBER activity. Whereas AT2 has the same enantiospecificity as the PLR from *L. usitatissimum*, AT2 doesn't show enantioselectivity for the PINO enantiomers but slightly prefers (-)-LARI in contrast to (+)-LARI (Fig. 35). AT3 is more effective as a PCBER but shows also PLR activity and therefore is the first cloned PCBER with PLR activity, too. It prefers (+)-PINO when acting as PLR.

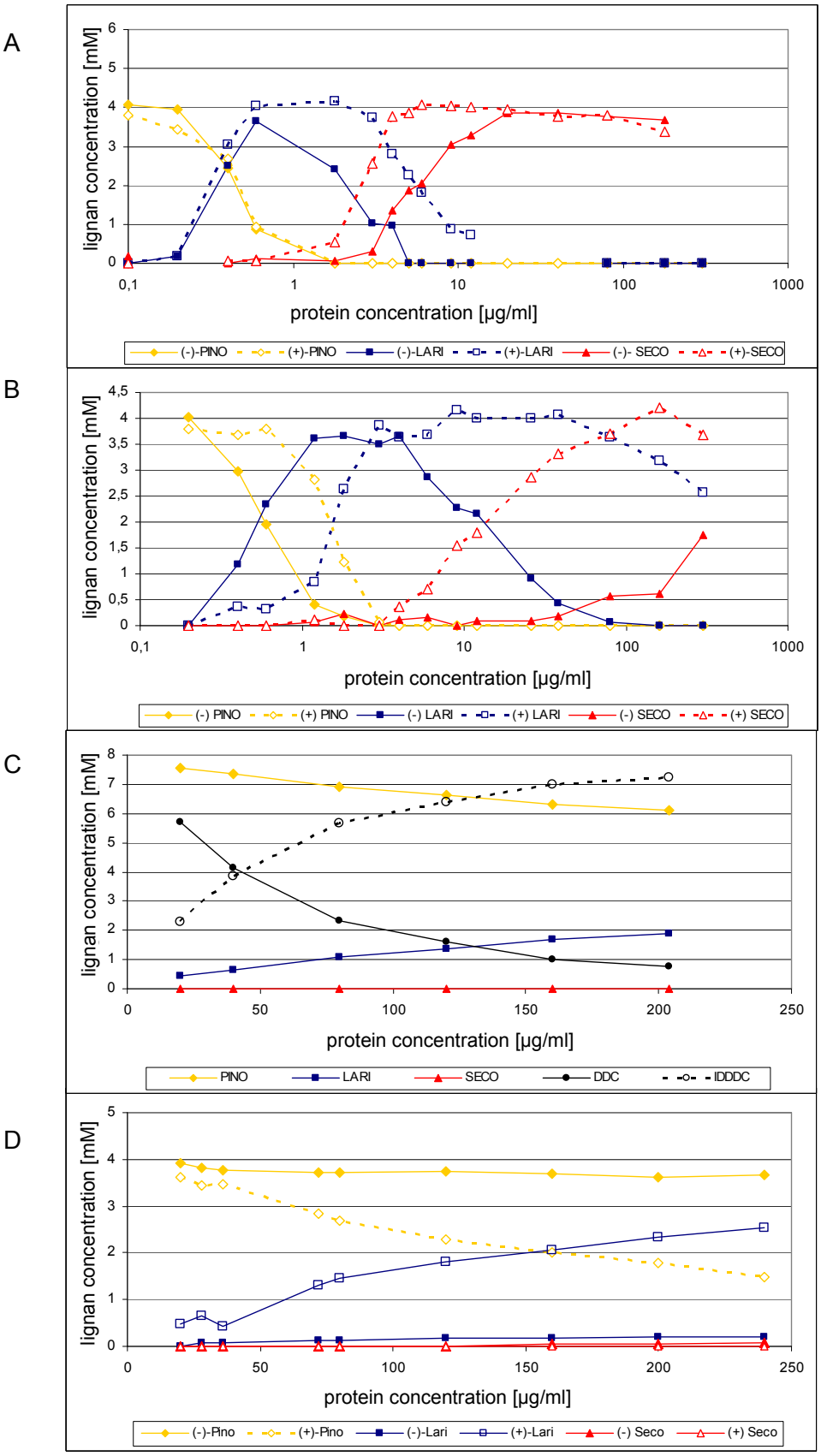


Fig. 35: Enantiomeric composition of lignans in assays with racemic PINO as substrate with purified AT1 (A), AT2 (B); dependence of PLR and PCBER activity from the concentration of AT3 in the assay (C); enantiomeric composition of PINO, LARI and SECO in PLR assays with AT3 and racemic PINO as substrate (D) (One example of three independent determinations is shown.)

AT4, AT 6 and AT7 show only poor PLR and PCBER activities. Therefore they may have other not yet identified functions.
Recently new members of the PIP-family were identified: Pterocarpan reductases (PTRs) which are like IFRs involved in isoflavone biosynthesis (Akashi et al. 2006); eugenol synthases (EGSs) and isoeugenol synthases (IGSs), which use coniferyl alcohol acetate as substrate (Koeduka et al. 2006); leucoanthocyanidin reductases (LARs) involved in the biosynthesis of flavonoids (Pfeiffer et al. 2006). Sequences of 3,8-divinylprotochlorophyllide *a*-8-vinyl-reductases (DVRs) which are involved in the biosynthesis of chlorophyll a and UDP-glucose/galactose 4-epimerases (GALEs), enzymes which are involved in carbohydrate metabolism in plants and other organisms have more distant similarity to the members of the PIP-family. They could be the progenitors for the whole family.
A phylogenetic tree for the PIP-family was calculated with the MEGA software (Fig. 36). GALE, VRL-AT9 and DVR count as outgroup. LARs form an own cluster. All other proteins form two main clusters which can be divided in two subclusters each. One of the main clusters contains the PLRs and the subcluster of EGS and IGS. The second main cluster consists of the cluster of IFRs and the cluster of PCBERs and PTRs. Therefore, LARs and all other reductases have a common ancestor. Then the groups of PLR/IGS/EGS and PCBER/IFR were developed which were separated into PLR and IGS/EGS and PCBER and IFR, respectively. Interestingly, the group of PLRs is divided in two subclusters. One contains PLRs from the Coniferopsida, the other of Magnoliopsida. The Magnoliopsida PLRs build two clusters. One cluster contains PLRs with enantioselectivity for (+)-PINO. A broad variety in enantiospecificity can be found for the PLRs of the second cluster. Two gene copies for encoding PLRs had to be in the genome of the *Linum* ancestor because the PLRs of *L. usitatissimum* are closer related to other *Linum* species than to each other. The same is true for the PLRs in the Coniferopsida. The clear separation of the PLR clusters of Magnoliopsida and Coniferopsida indicates a *plr* gene duplication during development of these orders. The separation of PCBERs and PLRs in the orders and the presence of PLRs and PCBERs within one plant species indicate that PCBERs and PLRs were already developed in the progenitors of the orders. Therefore, the progenitor of PLRs and PCBERs has to be present in primitive plants. This progenitor could show already enantiospecificity because at least the PCBER AT3 of *A. thaliana* prefers (+)-PINO in assays for PLR activity. Therefore, enantiospecificty is an old invention in evolution of the PIP-family. On the other hand, PLR-AT1, PLR-AT2, PLR-Lp1 und PLR-Lu1 show a high variability in enantiospecificity from not enantiospecific (PLR-AT1) to highly enantiospecific (PLR-Lu1). This indicates that enantiospecificity can change and get lost during evolution. This is in contrast to Gang et al. (1999) who predicted the PCBERs as ancestors for the enantiospecific PLRs and IFRs because they are not enantiospecific.

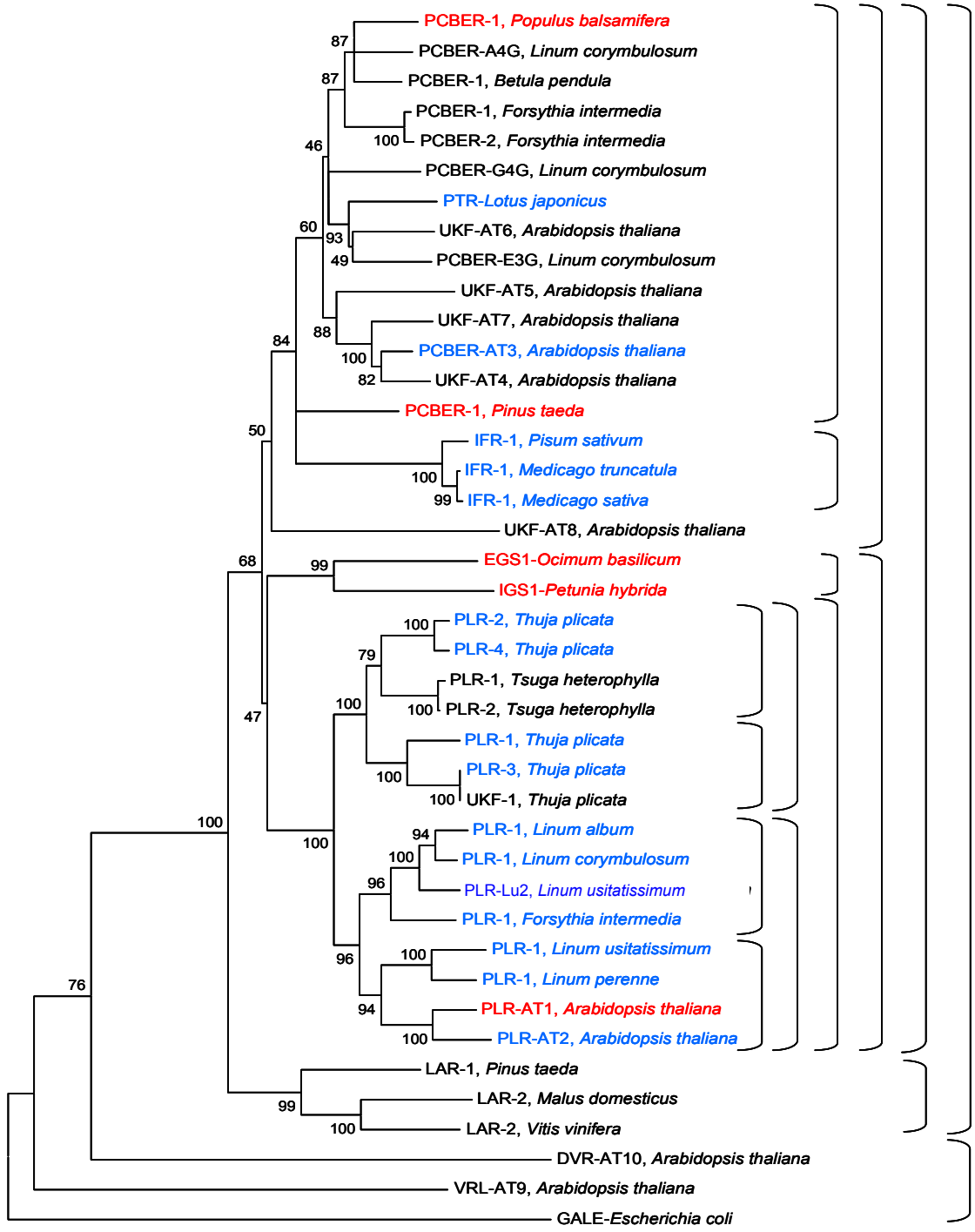


Fig. 36: Phylogenetic tree of pinoresinol-lariciresinol reductases and related proteins, calculated with the MEGA software (method: Neighbour Joining, Bootstrap: 1000); PLR=pinoresinol-lariciresinol reductase, IFR=isoflavone-reductase, PCBER=phenylcoumaran benzylic ether reductase. PTR=pterocarpan reductase, EGS=eugenol synthase, IGS=isoeugenol synthase; LAR=leucoanthocyanidin reductase, DVR=3,8-divinyl protochlorophyllide *a*-8-vinyl reductase, GALE=UDP-glucose/galactose 4-epimerase, UKF=unknown function;
red: not enantiospecific, blue: enantiospecific, black: enantiospecificity not known

The ancestor of *Linum* species probably contained at least two copies of *plr* genes. One copy encoded a PLR with specificity for (+)-PINO whereas the PLRs developed from the other copy show high variability of enantiospecificity. We will clone more cDNAs encoding PLRs from *Linum* species to proof this assumption.
The comparison of the enantiospecificity of PLRs acting in the lignan metabolism and the type of lignans accumulated leads to the following statements. The predominant enantiomers of furofuran, furan and dibenzylbutane lignans vary whereas most dibenzylbutyrolactone lignans are levororatory (Umezawa 2003). Dextrorotatory dibenzylbutyrolactone lignans were only detected in Thymeleaceae plant species and in *Selaginella doederleinii*. Aryltetralin lignans found in nature so far are all levorotatory. E. g. (+)-secoisolariciresinoldiglucoside is found in seeds of *L. usitatissimum* whereas the flowering plant contains lignans up to yatein which are levorotatory (Schmidt et al. 2006). This opens the question about the importance of the absolute configuration for the biosynthesis of more complex lignans like the arylteralines PTOX and 6MPTOX or the arylnaphthalene JusB. It seems that the enzymes necessary for the derivatisation to the complex lignans only accept lignans with R,R-configurations at C-atoms 8,8'. The more complex lignans are probably of higher ecological value than the simple lignans. Therefore, the maintenance of the enantiospecificity for the formation of (-)-secoisolariciresinol by the (+)-pinoresinol selective PLRs could be of ecological advantage in evolution.

5.5. Conclusion

Pinoresinol-lariciresinol reductases (PLRs) are involved in the early steps of the lignan biosynthesis. Most of them but not all are enantiospecific. We could figure out that the enantiomeric purity of the lignans is determined in the steps catalysed by PLR in *Linum* species. Therefore, the focus of our studies was to understand the evolution of this enzyme and its enantiospecificity.
We cloned several genes encoding PCBERs and PLRs from *Linum* species and *Arabidopsis thaliana*. The proteins were heterologously expressed, purified and investigated for PCBER and PLR activity. The PLR AT1 of *A. thaliana* is the first PLR which shows PCBER activity, too. The PCBER AT3 of *A. thaliana* is the first PCBER which shows PLR activity, too, thus indicating the evolutionary relationship between these reductases. AT3 prefers (+)-PINO if acting as PLR indicating that the capability for the enantiospecificity is present in PCBERs as well as in PLRs and IFRs.
A first phylogenetic tree of PLRs but also related reductases indicates that the capability for enantiospecificity was established early in evolution. A gene duplication of the PLR gene from the *Linum* ancestor has occurred during development to the different *Linum* species. One form which is specific for (+)-PINO kept its enantiospecificity during evolution. The PLRs which were evolved from the second gene copy show a high variability in enantiospecificity. These assumptions have to be proven by completion of the phylogenetic tree with PLR sequences from more *Linum* species. Three amino acids responsible for the enantiospecificity of PLRs are identified by a mutagenesis approach.

Complex lignans only accumulate in plants or organs of *Linum* species if (-)-SECO (is an intermediate opening the question whether the enzymes involved in further derivatisation of the general structures accept only lignans with R,R configuration at C-atoms 8,8' like (-)-SECO.

6. Outlook

Most parts of the biosynthetic pathway of PTOX and 6MPTOX are not yet known. Especially from matairesinol only a few enzymes are characterised (Fig. 4). No genes are available. No regulatory genes are cloned so far. We have established cell culture systems with differential PTOX and 6MPTOX accumulation in *L. album*. Especially the elicitation with methyljasmonate or coronalon are a promising system to clone genes involved in the biosynthesis of the aryltetralin lignans by molecular biological methods like cDNA-AFLP (Goossens 2003). Therefore, we will apply cDNA-AFLP to clone the genes involved in lignan biosynthesis in cooperation with A. Goossens (VIB, Gent, Belgium). The candidate genes will be checked for their involvement in PTOX/6MPTOX biosynthesis by the ihpRNAi which we have established for *Linum* hairy roots.

We have set up a phylogenetic tree of PLRs and related proteins. This gives first insights into the evolution of PIP-reductases and their enantiospecificity. But the phylogenetic tree will be completed by cloning further members of the PIP-family from *Linum* species, but also mosses and ferns which contain lignans to get more insight in the progenitors of this reductase family.

The conformation of lignans at C-atoms 8,8' seems to be important for the fate of the simple lignans as precursors for the biosynthesis of more complex lignans. As an example, seeds of *L. usitatissimum* contain 99% (+)-secoisolariciresinoldiglucoside whereas flowering plants accumulate more derived lignans up to (-)-yatein which show opposite enantiomeric composition. To test our assumption we will genetically exchange the enantiomeric composition of SECO in seeds and leaves of *L. usitatissimum* in cooperation with E. Lainé (Chartres, France). The group of E. Lainé has cloned the promotor region of the (-)-PINO specific PLR of *L. usitatissimum*. The specificity of the promotor region for gene expression in seeds was shown by transformation of *L. usitatissimum* with promotor-GUS-fusions (Hano et al. 2006). In one experiment the expression of the (+)-SECO forming PLR will be downregulated together with overexpression of the (-)-SECO forming PLR in seeds of *L. usitatissimum*. The fate of (-)-SECO will be followed in the seeds to check whether the enzymes for the formation of yatein are present in seeds as well as in leaves. In a second experiment the expression of the (-)-SECO forming PLR will be downregulated together with overexpression of the (+)-SECO forming PLR in leaves. The fate of the (+)-SECO in leaves will be followed to check whether the enzymes involved in yatein synthesis can accept (+)-SECO as substrate.

7. References

Akashi T, Koshimizu S, Aoki T, Ayabe S (2006) Identification of cDNAs encoding pterocarpan reductase involved in isoflavan phytoalexin biosynthesis in *Lotus japonicus* by EST mining. FEBS Lett. 580, 5666-5670

Alfermann AW, Petersen M, Fuss E (2003) Production of natural products by plant cell biotechnology: results, problems and perspectives. In: Plant Tissue Culture, 100 years since Gottlieb Haberlandt (M. Laimer, W. Rücker, Eds.), Springer, pp. 153-166

Baba A, Kawamura N, Makino H, Ohta Y, Taketomi S, Sohda T (1996) Studies on disease-modifying antirheumatic drugs: Synthesis of novel quinoline and quinazoline derivatives and their anti-inflamatory effect. J. Med. Chem.39, 5176-5182

Berim A, Spring O, Conrad J, Maitrejean M, Boland W, Petersen M (2005) Enhancement of lignan biosynthesis in suspension cultures of *Linum nodiflorum* by coronalone, indanoy-isloleucine and methyl jasmonate. Planta 222, 769-776

Broomhead AJ, Dewick PM (1991) Biotransformation of *Podophyllum* lignans in cell suspension cultures of *Forsythia intermedia*. Phytochemistry 30: 1511-1517

Chattopadhay S, Mehra RS, Srivastava Ak, Bhojwani SS, Bisaria VS (2003) Effect of major nutrients on podophyllotoxin production in *Podophyllum hexandrum* suspension cultures. Appl. Microbiol. Biotechnol. 60, 541-546

Chen CC, Hsin WC, Ko FN, Huang YL, Ou JC, Teng CM (1996) Antiplatelet arylnaphthalide lignans from *Justica procumbens*. J. Nat. Prod. 59, 1149-1150

Da Silva R, de Souza GHB, da Silva AA, de Souza VA, Peirera AC, Royo VD, Silva MLAE, Donate PM, Araujo ALSD, Carvalho JCT, Bastos JK (2005) Synthesis and biological activity evaluation of lignan lactones derived from (-)-cubebin. Bioorg. Med. Chem. Lett. 15, 1033-1037

Davin LB, Wang H-B, Crowell AL, Bedgar DL, Martin DM, Sarkanen S, Lewis NG (1997) Stereoselective bimolecular phenoxy radical coupling by an auxiliary (dirigent) protein without an active center. Science 275, 362-366.

Davin LB, Lewis NG (2003) An historical perspective on lignan biosynthesis: Monolignol, allyphenol and hydroxycinnamic acid coupling and downstream metabolism. Phytochem. Rev. 2, 257-288

Dinkova-Kostova AT, Gang DR, Davin LB, Bedgar DL, Chu A, Lewis NG (1996) (+)-Pinoresinol/(+)-lariciresinol reductase from *Forsythia intermedia*: protein purification, cDNA

cloning, heterologous expression and comparison to isoflavone reductase. J. Biol. Chem. 271, 29473-29482.

Dixon RA, Reddy MSS (2003) Biosynthesis of monolignols. Genomic and reverse genetic approaches. Phytochem. Rev. 2, 289-306

Fujita Y, Tabata M, Nishi A, Yamada Y (1998) New medium and production of secondary compounds with the two-staged culture method. In: Fujiwara A (ed.) Plant Tissue Culture 1982. Mazuren, Tokyo, pp. 399-400

Fujita M, Gang DR, Davin LB, Lewis NG (1999) Recombinant pinoresinol-lariciresinol reductases from Western Red Cedar (*Thuja plicata*) catalyze opposite enantiospecific conversions. J. Biol. Chem. 274, 618-627

Gang DR, Kasahara H, Xia ZQ, Vander Mijnsbrugge K, Bauw G, Boerjan W, Van Montagu M, Davin LB, Lewis NG (1999) Evolution of plant defense mechanism. J. Biol. Chem. 274, 7516-7527

Gertsch J, Tobler RT, Brun R, Sticher O, Heilmann S (2003) Antifungal, antiprotozoal, cytotoxic and piscicidal properties of justicidin B and a new arylnaphthalide lignan from *Phyllanthus piscatorum*. Planta Med. 69, 420-424

Giri A, Narasu ML (2000) Production of podophyllotoxin from *Podophyllum hexandrum*: a potential natural product for clinically useful anticancer drugs. Cytotechnology 34, 17-26

Goossens A, Hakkinen ST, Laakso I, Seppanen-Laakso T, Biondi S, De Sutter V, Lammertyn F, Nuutila AM, Soderlund H, Zabeau M, Inze D, Oksman-Caldenty KM (2003) A functional genomis approach toward the understanding of secondary metabolism in plant cells. Proc. Nat. Acad. Sci. 100, 8595-8600

Guillon S, Trémouillaux-Guiller J, Pati PK, Rideau M, Gantet P (2006) Hairy root research: recent scenario and exciting prospects. Commentary. Curr. Op. plant Biol. 9, 341-346

Hano C, Martin I, Fliniaux O, Legrand B, Gutierrez L, Arroo RRJ, Mesnard F, Lamblin F, Lainé E (2006). Pinoresinol-lariciresinol reductases gene expression and secoisolariciresinol diglucoside accumulation in developing flax (*Linum usitatissimum*) seeds. Planta 224: 1291-1301

Huang RL, Huang YL, Ou JC, Chen CC, Hsu FL, Chang C (2003) Screening of 25 compounds isolated from *Phyllanthus* species for anti-human hepatitis B virus *in vitro*. Phytother. Res. 17, 449-453

Humphreys JM, Chapple C (2002) Rewriting the lignin roadmap. Curr. Op. Plant Biol. 5, 224-229

Imbert TF (1998) Discovery of podophyllotoxin. Biochimie 80, 207-222

Ionkova II, Goermova BP, Vasilev NP,Fuss E (2007) Lignan production in cell cultures of *Linum tauricum ssp. tauricum.* (Phytologia Balcanica, im Druck)

Khanna P, Jain SC (1973) Isolation and idendification of sesamin from *Sesamum indicum* tissue culture. Curr. Sci. 42: 253-254

Koeduka T, Fridman E, Gang DR, Vassao DG, Jackson BL, Kish CM, Orlova I, Spassova SM, Lewis NG, Noel JP, Baiga TJ, Dudareva N, Pichersky E (2006) Eugenol and isoeugenol, characteristic aromatic constituents of spices, are biosynthesized via reduction of a coniferyl alcohol ester. Proc. Natl. Acad. Sci. 27, 10128-10130

Konuklugil B, Fuss E, Alfermann AW (2005): Isolation of the lignan 6-methoxy-podophyllotoxin from *Linum boissieri* and biosynthesis of lignans in this species. Pharmaceutical Biology 43, 737-739

Konuklugil B, Ionkova I, Vasilev N, Schmidt TJ, Windhövel J, Fuss E, Alfermann AW (2007): Lignans from *Linum* species of sections Syllinum and Linum. Natural Product Research 21, 1-6

Kranz K, Petersen M (2003) *β*-Peltatin 6-*O*-methyltransferase from cell suspension cultures of *Linum nodiflorum*. Phytochemistry 64, 453-458

Kutchan T, Diettrich H, Bracher D, Zenk MH (1991) Enzymology and molecular biology of alkaloid biosynthesis. Tetrahedron 47, 5945-5954

Larkin P, Scowcroft W (1981) Somaclonal variation – a novel source of variability from cell culture for plant improvement. Theor. Appl. Genet. 60, 197-214

MacRae WD, Hudson JB, Towers GH (1989) The antiviral action of lignans. Planta Med. 55, 531-535

Messner B, Boll M (1993) Elicitor-mediated induction of enzymes of lignan biosynthesis and formation of lignin-like material in a cell suspension culture of spruce (*Picea abies*). Plant Cell Tiss. Org. Cult. 34, 261-269

Min T, Kasahara H, Bedgar DL, Youn B, Lawrence PK, Gang DR, Halls SC, Park H, Hilsenbeck JL, Davin LB, Lewis NG, Kang C (2003) Crystal structures of pinoresinol-

lariciresinol and phenylcoumaran benzylic ether reductases and their relationship to isoflavone reductases. J. Biol. Chem. 278, 50714-50723

Mimura A, Takahara Y, Ichikawa A & Osawa T (1987) Lignan compounds and their manufacture with tissue culture of *Sesamum indicum*. JPN Kokai Tokkyo Koho:JP 87-40096 (in Japanese); cited from Ogasawara T, Chiba K, Tada M (1998) XIX *Sesamum indicum* L. (Sesame): *In vitro* culture, and the production of naphthoquinone and other secondary metabolites. In: Bajaj Y.P.S. (ed), Biotechnology in Agriculture and Forestry, Vol. 41 (pp. 366-393). Springer Verlag, Berlin Heidelberg

Mohagheghzadeh A, Schmidt TJ, Alfermann AW (2002) Arylnaphthalene lignans from in vitro cultures of *Linum austriacum*. J. Nat. Prod. 65 (1), 69-71

Mohagheghzadeh A, Hemmati S, Mehregan I, Alfermann AW (2003) *Linum persicum*: Lignans and plancement in Linaceae. Phytochem. Rev. 363-369

Moinuddin SGA, Youn B, Bedgar DL, Costa MA, Helms GL, Kang C, Davin LB, Lewis NG (2006) Secoisolariciresinol dehydrogenase: mode of catalysis and stereospecificity of hybride transfer in *Podophyllum peltatum*. Org. Biomol. Chem., 4, 808-816

Molog GA, Empt U, Kuhlmann S, Van Uden W, Pras N, Alfermann AW, Petersen M (2001) Deoxypodophyllotoxin 6-hydroxylase, a cytochrome P450 monooxygenase from cell cultures of *Linum flavum* involved in lignan biosynthesis. Planta 214, 288-294.

Moraes-Cerdeira RM, Burandt Jr CL, Bastos JK, Dhammika Nanayakkara NP & McChesney JD (1998) *In vitro* propagation of *Podophyllum peltatum*. Planta Med. 64: 42-45

Moss GP (2000) Nomenclature of lignans and neolignans (IUPAC Recommendations 2000). Pure Appl. Chem. 72, 1493-1523

Muranaka T, Miyata M, Ito K, Tachibana S (1998) Production of podophyllotoxin in *Juniperus chinensis* callus cultures treated with oligosaccharides and a biogenetic precursos. Phytochemistry 49, 491-496

Ogasawara T, Chiba K, Tada M (1998) *Sesamum indicum* L. (sesame): *in vitro* culture, and the production of naphthoquinone and other secondary metabolites. In: Bajaj Y.P.S. (ed), Biotechnology in Agriculture and Forestry, Vol. 41 (pp. 366-393). Springer Verlag, Berlin Heidelberg

Okunishi T, Sakakibara N, Suzuki S, Umezawa T, Shimada M (2004) Stereochemistry of matairesinol formation by *Daphne* secoisolariciresinol dehydrogenase. J. Wood Sci. 50, 77-81

Oostdam A, Mol JMN, van der Plas HW (1993) Establishment of hairy root cultures of *Linum flavum* producing the lignan 5-methoxypodophyllotoxin. Plant Cell Rep. 12, 474-477

Park S-U, Facchini PJ (2000) *Agrobacterium rhizogenes*-mediated transformation of opium poppy, *Papaver somniferum* L. and california poppy, *Eschscholzia californica* Cham. root cultures. J. Exp. Bot. 15, 1005-1016

Petersen M, Alfermann AW (2001) The production of cytotoxic lignans by plant cell cultures. Appl. Microbiol. Biotechnol. 55, 135-142

Petersen M, Szabo E, Meinhard J, Karwatzki B, Gertlowski C, Kempin B, Fuss E (1995): Biosynthesis and accumulation of rosmarinic acid in suspension cultures of *Coleus blumei*. Plant Cell Tissue Organ Culture 43: 89-93

Pfeiffer J, Kühnel C, Brandt J, Duy D, Punyasiri PAN, Forkmann G, Fischer TC (2006) Biosynthesis of flavan 3-ols by leucoanthocyanidin 4-reductaeses and anthocyanidin reductaese in leaves of grapes (*Vitis vinifera* L.), apple (*Malus domestica* Borkh.) and other crops. Plant Physiol. Biochem. 44, 323-334

Phyton Inc. (2002) Phyton expands commercial partnership with Bristol-Meyer Sqibb for paclitaxel supply. Press release July 10, 2002

Rahman MMA, Dewick PM, Jackson DE, Lucas JA (1990) Biosynthesis of lignans in *Forsythia intermedia* cell cultures. Phytochemistry 29: 1841-1846

Rao YK, Fang SH, Teng YM (2006) Anti-inflammatory activities of constituents isolated from *Phyllantus polyphyllus*. J. Ethnopharmacol. 16, 103, 181-186

Rickard-Bon SE, Thompson LU (2003) The role of flaxseed lignans in hormone-dependant and independent cancer. In: Flax, the genus *Linum*, Muir AD and Westcott ND (Eds.), Taylor and Francis

Rischer H, Oresic M, Seppanen-Laakso T, Katajama M, Lammertyn F, Ardiles-Diaz W, van Montagu MCE, Inze D, Oksman-Caldenty KM, Goossens A (2006) Gene-to-metabolite networks for terpenoid alkaloid biosynthesis in *Catharanthus roseus* cells. Proc. Nat. Acad. Sci. 103, 5614-5619

Sakakibara N, Suzuki S, Umezawa T, Shimada M (2003) Biosynthesis of yatein in *Anthriscus sylvestris*. Org. Biomol. Chem. 1, 2474-2485

Schmitt J, Petersen M (2002) Influence of methyl jasmonate and coniferyl alcohol on pinoresinol and matairesinol accumulation in a *Forsythia* x *intermedia* suspension culture. Plant Cell Rep. 20, 885-889

Schmidt TJ, Hemmati S, Fuss E, Alfermann AW (2006): A combined HPLC-UV and HPLC/MS-MS method for the identification of lignans and its application to the lignans of *Linum usitatissimum* L. and *Linum bienne* L.. Phytochemical Analysis 17, 299-311

Schüler G, Mithöfer A, Baldwin IT, Berger S, Ebel J, Santos JG, Hermann G, Hölscher D, Kramell R, Kutchan TM, Maucher H, Schneider B, Stenzel I, Wasternack C, Boland W (2004) Coronalone: a powerful tool in plant stress physiology. FEBS Lett. 563, 17-22

Seidel V, Windhövel J, Eaton G, Alfermann AW, Arroo RRJ, Medarde M, Petersen M, Woolley JG (2002) Biosynthesis of podophyllotoxin in *Linum album* cell cultures, Planta 215, 1031-1039.

Smollny T, Wichers H, Kalenberg S, Shasavari A, Petersen M, Alfermann AW (1998) Accumulation of podophyllotoxin and related lignans in cell suspension cultures of *Linum album*. Phytochemistry 48, 975-979

Stafford HA (2000) In: Evolution of Metabolic Pathways, Romeo JT, Ibrahim R, Varin L, De Luca V (Eds.), Elsevier Amsterdam, pp. 25-54

Suzuki S, Umezawa T, Shimada M (2002) Stereochemical diversity in lignan biosynthesis of *Arctium lappa* L. Biosci., Biotechnol., Biochem. 66, 1262-1269

Szabo E, Thelen A, Petersen M (1999) Fungal elicitor preparation and methyl jasmonate enhance rosmarinic acid accumulation in suspension cultures of *Coleus blumei*. Plant Cell Rep. 18, 485-489

Takaku N, Choi D-H, Mikame K, Okunushi T, Suzuki S, Ohashi H, Umezawa T, Shimada M (2001) Lignans of *Chamaecyparis obtusa*. J. Wood Sci. 47, 476-482

Umezawa T (2003) Diversity in lignan biosynthesis. Phytochem. Rev. 3, 371-390

Van Uden W, Pras N, Homan B, Malingé TM (1991) Improvement of the production of 5-methoxypodophyllotoxin using a new selected root culture of *Linum flavum* L.. Plant Cell Tiss. Org. Cult. 27: 115-121

Van Uden W, Homann B, Woerdenbag HJ, Pras N, Malingré TM, Wichers HJ, Harkes M (1992) Isolation, purification and cytotoxicity of 5-methoxypodophyllotoxin, a lignan from a root culture of *L. flavum*. J. Nat. Prod. 55, 102–110

Van Uden W, Bouma AS, Walker JFB, Middel O, Wichers HJ, De Waard P, Woerdenbag HJ, Kellogg RM, Pras N (1995) The production of podophyllotoxin and its 5-methoxy derivative

through bioconversion of cyclodextrin-complexed desoxypodophyllotoxin by plant cell cultures. Plant Cell Tiss. Org. Cult. 42: 73-79

Vasilev N, Elfahmi, Bos R, Kayser O, Momekov G, Konstantinov S, Ionokova I (2006) Production of jucticidin B, a cytotoxic arylnaphthalene lignan from genetically transformed root cultures of *Linum leonii*. J. Nat. Prod. 69, 1014-1017

Verpoorte R, Alfermann AW (Eds.) (2000) Metabolic engineering of secondary metabolism. Dordrecht: Kluwer Academic Publishers

Verpoorte R, Memelink J (2002) Engineering secondary metabolite production in plants. Curr. Op. Plant Biotech. 13, 181-187

Watson JM, Fusaro AF, Wang M-B, Waterhouse P (2005) RNA silencing platforms in plants. FEBS Lett. 579,5982-5987

Wesley SV, Helliwell CA, Smith NA, MingBo W, Rouse DT, Liu Q, Gooding PS, Singh SP, Abbott D, Stoutjesdijk PA, Robinson SP, Gleave AP, Green AG, Waterhouse PM (2001) Construct design for efficient, effective and highthoughput gene silencing in plants. Plant J. 27 (6), 581-590

Xia ZQ, Costa MA, Péllisier H, Davin LB, Lewis NG (2001) Secoisolariciresinol dehydrogenase purification, cloning and functional expression. J. Biol. Chem. 276, 12614-12623

Youn BY, Moinuddin SGA, Davin LB, Lewis NG, Kang CH (2005) Crystal structures of apo-form and binary/ternary complexes of *Podophyllum* secoisolariciresinol dehydrogenase, an enzyme involved in formation of health-protecting and plant defense lignans. J. Biol. Chem. 280, 12917-12926

Zenk MH, Al-Shagi H, Ahrens H, Stöckigt J, Weiler EW, Deus B (1977) Formation of indole alkaloids serpentine and ajmalicine in cell suspension cultures of Catharanthus roseus. In: Barz W, Reinhard E, Zenk MH (eds.) Plant tissue culture and its bio-technologicl application. Springer, Berlin, pp. 27-43

Zenk MH (1978) The impact of plant culture on industry. In: Proceedings of the 4th International Congress of Plant Tissue and Cell Culture, University of Calgary, Canada, pp. 1-13

Zenk MH (1991) chasing the enzymes of secondary metabolism: Plant cell cultures as a pot of gold. Phytochemistry 30, 3861-3863

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